

FORM PTO 1390 (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES P E REVEL=15					
DESIGNATED/ELECTED OFFICE (DO/EO/US)				U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
CONCERNING A FILING UNDER 35 U.S.C. 371 JAN 10 2000				09/462416	
INTERNATIONAL APPLICATION NO.	PCT/IL98/00321	INTERNATIONAL FILING DATE	9 July 1998	PATENT & TRADEMARK	PRIORITY DATE CLAIMED
TITLE OF INVENTION <b>CHIMERIC INTERLEUKIN-6 SOLUBLE RECEPTOR/LIGAND PROTEIN, ANALOGS THEREOF AND...</b>					
APPLICANT(S) FOR DO/EO/US <b>Michel REVEL et al.</b>					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/>	This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.				
2. <input type="checkbox"/>	This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.				
3. <input checked="" type="checkbox"/>	This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).				
4. <input checked="" type="checkbox"/>	A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.				
5. <input checked="" type="checkbox"/>	A copy of the International Application as filed (35 U.S.C. 371(c)(2))				
	a. <input type="checkbox"/>	is transmitted herewith (required only if not transmitted by the International Bureau).			
	b. <input checked="" type="checkbox"/>	has been transmitted by the International Bureau.			
	c. <input type="checkbox"/>	is not required, as the application was filed in the United States Receiving Office (RO/US).			
6. <input type="checkbox"/>	A translation of the International Application into English (35 U.S.C. 371(c)(2)).				
7. <input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))				
	a. <input type="checkbox"/>	are transmitted herewith (required only if not transmitted by the International Bureau).			
	b. <input type="checkbox"/>	have been transmitted by the International Bureau.			
	c. <input type="checkbox"/>	have not been made; however, the time limit for making such amendments has NOT expired.			
	d. <input checked="" type="checkbox"/>	have not been made and will not be made.			
8. <input type="checkbox"/>	A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).				
9. <input type="checkbox"/>	An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).				
10. <input type="checkbox"/>	A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).				
Items 11. to 16. below concern document(s) or information included:					
11. <input type="checkbox"/>	An Information Disclosure Statement under 37 CFR 1.97 and 1.98.				
12. <input type="checkbox"/>	An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.				
13. <input checked="" type="checkbox"/>	A <b>FIRST</b> preliminary amendment.				
	<input type="checkbox"/>	A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.			
14. <input type="checkbox"/>	A substitute specification.				
15. <input type="checkbox"/>	A change of power of attorney and/or address letter.				
16. <input checked="" type="checkbox"/>	Other items or information:				
<ol style="list-style-type: none"> <li>1. A courtesy copy of the specification as originally filed with sequence.</li> <li>2. A courtesy copy of the first page of the International Publication (WO99/02552).</li> <li>3. A courtesy copy of the International Search Report.</li> <li>4. A courtesy copy of the International Preliminary Examination Report.</li> <li>5. Formal drawings, 15 sheets, figures 1-12.</li> </ol>					

17.  The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5) ) :**

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$970.00  
 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$840.00  
 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$760.00  
 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$670.00  
 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$96.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$ 840.00

Surcharge of \$130.00 for furnishing the oath or declaration later than  20  30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	37 - 20 =	17	X \$18.00	\$ 306.00
Independent claims	1 - 3 =	0	X \$78.00	\$ -0-
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 1,276.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$
<b>SUBTOTAL =</b>				\$ 1,276.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$
<b>TOTAL NATIONAL FEE =</b>				\$ 1,276.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$
<b>TOTAL FEES ENCLOSED =</b>				\$ 1,276.00
				Amount to be: refunded
				\$ charged

a.  A check in the amount of \$ 1,276.00 to cover the above fees is enclosed.

b.  Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c.  The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4035. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

SIGNATURE

**BROWDY AND NEIMARK, P.L.L.C.  
624 Ninth Street N.W., Suite 300  
Washington, D.C. 20001**

\_\_\_\_\_  
**Norman J. Latker**

NAME

19,963

REGISTRATION NUMBER

Date of this submission: January 10, 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit:
	)	
Michel REVEL et al.	)	
	)	
IA No.: PCT/IL98/00321	)	
	)	Washington, D.C.
IA Filed: 9 July 1998	)	
	)	
U.S. App. No.:	)	
(Not Yet Assigned)	)	
	)	January 10, 2000
National Filing Date:	)	
(Not Yet Received)	)	
	)	
For: CHIMERIC INTERLEUKIN-6...	)	Docket No.: REVEL=13

PRELIMINARY AMENDMENT

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and prior to calculation of the filing fee, kindly amend as follows:

IN THE SPECIFICATION

Page 1, after the title please insert the following paragraph:

--CROSS REFERENCE TO RELATED APPLICATION

The present application is the national stage under 35 U.S.C. 371 of PCT/IL98/00321, filed 9 July 1998. --.

Page 13, line 7, after "(one-letter code)" insert  
--(SEQ ID NO:7)--.

Page 15, line 1, after "(one-letter code)" insert  
--(SEQ ID NO:8)--.

IN THE CLAIMS

Claim 6, line 1, delete "any one of claims 1-4", and

insert therefor --claim 1--.

Claim 7, line 1, delete "any one of claims 1, 2 and 5",  
and insert therefor --claim 1--.

Claim 9, line 1, delete "any one of claims 1-8", and  
insert therefor --claim 1--.

Claim 12, line 2, delete "any one of claims 1-11", and  
insert therefor --claim 1--.

Claim 14, line 2, delete "any one of claims 1-11", and  
insert therefor --claim 1--.

Claim 15, line 2, delete "any one of claims 1-11", and  
insert therefor --claim 1--.

Claim 16, line 2, delete "any one of claims 1-11", and  
insert therefor --claim 1--.

Claim 17, lines 2-3, delete "any one of claims 1-11",  
and insert therefor --claim 1--.

Claim 20, line 1, delete "claim 17-19", and insert  
therefor --claim 17--.

Claim 21, line 1, delete "any one of claims 17-20", and  
insert therefor --claim 17--.

Claim 22, line 1, delete "any one of claims 17-20", and  
insert therefor --claim 17--.

Claim 23, lines 1-2, delete "any one of claims 17-22",  
and insert therefor --claim 17--.

Claim 27, lines 1-2, delete "any one of claims 1-11",  
and insert therefor --claim 1--.

Claim 29, lines 1-2, delete "any one of claims 1-11",  
and insert therefor --claim 1--.

Claim 30, lines 1-2, delete "any one of claims 1-11",  
and insert therefor --claim 1--.

Claim 31, lines 1-2, delete "any one of claims 1-11",

00000000000000000000000000000000

and insert therefor --claim 1--.

Claim 32, lines 1-2, delete "any one of claims 1-11",  
and insert therefor --claim 1--.

Claim 33, line 2, delete "any one of claims 1-11", and  
insert therefor --claim 1--.

Claim 37, line 5, delete "any one of claims 33-36", and  
insert therefor --claim 33--.

If, inadvertently, a proper multiple dependent claim has  
not been amended to reduce it to single dependency, please amend  
it to be dependent solely on the first-mentioned claim, or, if  
that is not possible, please cancel the claim and notify the  
undersigned.

REMARKS

The present application is a national phase application  
of PCT/IL98/00321, filed July 9, 1998. A sequence listing and  
computer readable form (CRF) were filed in PCT/IL98/00321. While  
a paper copy sequence listing is included in the present  
application, filing of a new CRF should not be necessary.

The above amendments to the claims are being made in  
order to eliminate multiple dependency and for the purpose of  
reducing the filing fee. Please enter this amendment prior to  
calculation of the filing fee in this case.

Favorable consideration and allowance are earnestly  
solicited.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicant

By N - NL  
Norman J. Latker  
Registration No. 19,963

NJL:edg

Telephone No.: (202) 628-5197  
Facsimile No.: (202) 737-3528  
f:\filing\preliminary amendments\revel15.wpd

15/pcts

1

**CHIMERIC INTERLEUKIN-6 SOLUBLE RECEPTOR/LIGAND PROTEIN,  
ANALOGS THEREOF AND USES THEREOF**

5    **Field of the invention**

The present invention is generally in the field of interleukin-6 (IL-6) biological activities which are dependent on the agonistic action of soluble IL-6 receptor (sIL-6R). More specifically, the present invention concerns novel chimeric sIL-6R/IL-6 proteins constructed from the fusion of essentially the naturally occurring form of sIL-6R and IL-6, and biologically active analogs thereof, which are particularly useful for treating cancer, via inhibition of cancerous cell growth, for enhancing bone marrow transplantation, for treating liver disorders and other IL-6 related conditions.

15    **Background of the Invention and Prior Art**

Interleukin-6 (IL-6) is a well known cytokine whose biological activities are mediated by a membranal receptor system comprising two different proteins one named IL-6 Receptor (IL-6R or gp80) and the other gp130 (reviewed by Hirano et al, 1994). Soluble forms of IL-6R (sIL-6R), corresponding to the extracellular domain of gp80, are natural products of the human body found as glycoproteins in blood and in urine (Novick et al, 1990, 1992). An exceptional property of sIL-6R molecules is that they act as potent agonists of IL-6 on many cell types including human cells (Taga et al, 1989; Novick et al, 1992). This is due to the fact that even without the intracytoplasmic domain of gp80, sIL-6R is still capable of triggering the dimerization of gp130 in response to IL-6, which in turn mediates the subsequent IL-6-specific signal transduction and biological effects (Murakami et al, 1993). The active IL-6 receptor complex is in fact a hexameric structure formed by

two gp130 chains, two IL-6R and two IL-6 ligands (Ward et al, 1994; Paonessa et al, 1995), in which sIL-6R has two types of interaction with gp130 both of which are essential for the IL-6-specific biological activities (Halimi et al, 1995).

Treatment with sIL-6R results in an enhancement of the biological activities  
5 of IL-6 in many cell types. An example is tumor cells whose growth is inhibited to a greater extent by IL-6 when sIL-6R is added, such as murine myeloleukemic M1 cells (Taga et al, 1989), human breast carcinoma T47D cells (Novick et al, 1992) or human Non-small cell lung carcinoma cells (Ganapathi et al, 1996). IL-6 has anti-metastatic activities *in vivo* (Katz et al, 1995), sIL-6R and can also enhance  
10 such *in vivo* anti-tumor effects of IL-6 (Mackiewicz et al 1995). Another activity of IL-6 which is enhanced by sIL-6R addition, is the stimulation of hematopoietic stem cells to produce multilineage colonies (Sui et al, 1995). The present inventors have also observed that the survival of primary cultures of brain oligodendrocytes is supported by the sIL-6R and IL-6 combination (Oh, 1997), while IL-6 alone is  
15 poorly active in such cultures (Kahn and De Vellis, 1994). This finding indicates that IL-6, when combined with sIL-6R, can mimic the activity of other neurotropic cytokines such as Ciliary Neurotropic Factor (CNTF) or Leukemia Inhibitory Factor (LIF) which also act through gp130, as is also the case for IL-11 and Oncostatin M (Hirano et al, 1994).

20 In an attempt to provide a molecule which may combine the above noted functions of IL-6 and sIL-6R, there has recently been reported the production in recombinant yeast cells of a fusion protein between a truncated segment of the human IL-6R sequence and IL-6, linked by a glycine-rich linker (Fischer et al., 1997). This fusion protein includes essentially only the IL-6R cytokine receptor  
25 N-domain and the cytokine receptor C-domain, and thus lacks essentially all of the IL-6R immunoglobulin (Ig)-like domain, and the receptor pre-membrane region (the region between the C-domain and the transmembranal domain). As such it

represents a truncated form of the sIL-6R, this truncated sIL-6R in the fusion protein being linked via the above noted glycine-rich linker to essentially the whole mature form of IL-6. Besides lacking parts of the natural sIL-6R, this fusion protein by being produced in yeast cells, does not have the glycosylation pattern that such a  
5 fusion protein would have if it were produced in mammalian cells, in particular, e.g. in human cells. In fact, this yeast-produced fusion protein has a molecular weight of only about 57 kDa in contrast to a fusion product containing essentially all of the natural sIL-6R and IL-6 amino acid residues and being fully glycosylated in mammalian (e.g. human) cells, which has the expected molecular weight of about  
10 85 kDa (see Example 2 herein below).

The common experience in developing recombinant proteins which can be used for treating human patients has shown that it is important to remain as close as possible to the natural forms of the proteins, as they are found in the human body, in order to avoid triggering of antibodies and other side effects observed with  
15 non-natural recombinant products. For this reason, it has been advantageous to use recombinant mammalian cell systems to produce glycosylated proteins such as Interferon- $\beta$  or Granulocyte-colony stimulating factor (Chernajovsky et al, 1984, Holloway, 1994) in a chemical form as similar as possible to the natural human product. Bacteria or microorganisms such as, for example, yeasts, which do not  
20 glycosylate properly, also cause the wrong folding of the protein chains, leading to immunogenic reactions. This is particularly important in respect of IL-6 which is heavily modified posttranslationally by N- and O- glycosylation as well as by phosphorylation (Revel, 1989 for review), and in respect of the natural sIL-6R from human blood and urine which is a glycoprotein whose N-terminus and C-terminal  
25 amino-acids are constant and have been determined (Novick et al, 1990 and co-owned patents by the present inventors Nos. US 5,216,128 and its corresponding EP 413908 B1).

Accordingly, it would seem that the above noted previous fusion product between part of the sIL-6R and IL-6 has a number of possible drawbacks especially as regards its use for treating humans and this, due to the fact that it lacks part of the sIL-6R, as well as its production in yeasts which may provide for incorrect 5 glycosylation of the protein.

Heretofore, a fusion molecule comprising the natural sIL-6R found in human body fluids and the natural IL-6, and which is produced in human or other mammalian cells, has not been described.

It is therefore an aim of the present invention to provide such a fusion 10 molecule comprising the natural sIL-6R and the natural IL-6 (in any order) which is produced in mammalian cells.

It is another aim of the present invention to use such a fusion protein (sIL-6R/IL-6 chimera) to inhibit the growth of highly metastatic melanoma cells at very low concentrations, these cells being resistant to IL-6 or sIL-6R alone.

15 Yet another aim is to use such a fusion protein (the sIL-6R/IL-6 chimera) for the *in vivo* engraftment of human hematopoietic stem cells in bone marrow transplantation protocols.

It is a yet further aim of the present invention to use such a fusion protein in other IL-6 related disorders, e.g. liver conditions or neurological conditions.

20 A further aim of the invention is to provide pharmaceutical compositions which contain the above mentioned natural sIL-6R-natural IL-6 fusion protein (sIL-6R/IL-6 chimera) for the treatment of cancer, for use in bone marrow transplantation procedures, and for other IL-6 related disorders, e.g. liver conditions and neurological conditions.

25 Other aims and aspects of the present invention will be set forth or will arise from the following disclosure of the present invention.

### Summary of the Invention

In accordance with the present invention there have been produced a number of fusion proteins (chimeras) each comprising essentially all of the naturally occurring sIL-6R from human body fluids and essentially all of the mature form of the naturally occurring human IL-6, and each joined by short linker peptides which can be as short as 3 amino acid residues in length or longer, for example, 13 amino acid residues in length (see below and Examples 1 and 2). It should be noted, however, that in these fusion proteins the linker peptides may be omitted and the sIL-6R moiety may be directly linked to the IL-6 moiety. Since linkers representing non-natural amino acid sequences may be immunogenic epitopes eliciting antibodies in patients, it is preferable to have a directly fused sIL-6R/IL-6 chimera that has the desired biological activity, while at the same time there is minimized the risk of inducing such potentially deleterious antibody formation when such a chimera is administered.

The conservation of the entire sIL-6R sequence including the Ig-like domain as found in the naturally occurring molecule, as well as the proper glycosylation and other post-translational modifications introduced by human or mammalian cells when the above chimera is produced in such cells, are also important to reduce the potential immunogenicity of the chimeric protein product.

However, it is possible to use a very short linker of about three amino acids at the junction point between the sIL-6R and IL-6 moieties of the chimeric protein. Such a short linker would not be an immunogenic epitope. It is of course also possible to use longer linkers of up to about 30 amino acids to provide for separation between the two moieties but here care must be taken and biological efficacy and safety experiments must be performed to ensure that chimeric molecules with such linkers are not immunogenic.

In fact, it has been surprisingly shown in accordance with the present invention that such longer linkers are not essential for the activity of the chimeric protein indicating that proper folding of the chimera does not require a longer linker especially when essentially all of the naturally-occurring sequences of the sIL-6R and IL-6 moieties are incorporated into the chimeric molecule (see Example 3 and Fig. 5 which relate also to a comparison between a sIL-6R/IL-6 chimera having a very short (3 amino acids) linker and a similar chimera having a longer linker of 30 amino acids).

These fusion proteins or sIL-6R/IL-6 chimeras have been efficiently produced, in accordance with the present invention, in mammalian cell expression systems to yield glycosylated products having potent activity on tumor cells which are usually non-responsive to IL-6 or sIL-6R alone, and which were highly effective in ensuring the success of engraftment of human bone marrow transplanted cells (see below and Examples 1-4). In fact, in such bone marrow transplants, the sIL-6R/IL-6 chimeras were essential for the survival and proliferation of the transplanted non-committed pluripotential hematopoietic stem cells. Moreover, from the experimental results presented herein below, as well as from other analyses it arises that various analogs of the sIL-6R/IL-6 chimeric protein of the invention can be prepared, which have essentially the same biological activity of the sIL-6R/IL-6 chimera, these analogs being sIL-6R/IL-6 chimeras in which one or more amino acid residues have been deleted, added or substituted by others, the only limitation on such analogs being that they retain most of the naturally occurring sIL-6R and IL-6 sequence. For example, amino acid additions to the naturally occurring sIL-6R and IL-6 sequences are preferably limited to up to between about 20 amino acids, and preferably these additions are at the site of junction between the sIL-6R and IL-6, i.e. the linker molecule. Likewise, deletions from the sIL-6R and IL-6 sequences are preferably limited to up to between about

20-30 amino acids; and substitutions of amino acid residues in the sIL-6R and IL-6 sequences by other amino acid residues are preferably also limited to up to between about 20-30 amino acids. All of the aforesaid deletions, additions and substitutions are acceptable in accordance with the present invention when the so-modified 5 analogs that are obtained retain essentially the biological activity of the sIL-6R/IL-6 chimera composed of essentially the naturally-occurring sequences, and retain essentially the same glycosylation pattern of the chimera composed of essentially the naturally-occurring sequences when expressed in mammalian cells.

Accordingly, the present invention provides a chimeric glycosylated soluble 10 interleukin-6 receptor (sIL-6R)-interleukin-6 (IL-6) protein (sIL-6R/IL-6) and biologically active analogs thereof, comprising a fusion protein product between essentially all of the naturally occurring form of sIL-6R and essentially all of the naturally occurring form of IL-6, said sIL-6R/IL-6 and analogs thereof being glycosylated in a similar fashion to the glycosylation of naturally occurring sIL-6R 15 and IL-6.

Embodiments of the above chimeric protein of the invention include :

(i) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, wherein said sIL-6R is fused to IL-6 via a peptide linker molecule.

(ii) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, 20 as in (i) above, wherein said linker is a very short, non-immunogenic linker of about 3-4 amino acid residues.

(iii) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, as in (ii) above, wherein said linker is a tripeptide of the sequence E-F-M (Glu-Phe-Met).

(iv) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, 25 as in (i) above, wherein said linker is a peptide of 13 amino acid residues of

sequence E-F-G-A-G-L-V-L-G-G-Q-F-M

(Glu-Phe-Gly-Ala-Gly-Leu-Val-Leu-Gly-Gly-Gln-Phe-Met) (SEQ ID: NO 1).

5 (v) A chimeric sIL-6R/IL-6 protein, being the herein designated sIL-6R $\delta$ Val/IL-6 having a tripeptide linker of sequence E-F-M between the C-terminal Val-356 of sIL-6R and the N-terminal Pro-29 of IL-6, said chimeric protein having the sequence set forth in Fig. 3.

10 (vi) A chimeric sIL-6R/IL-6 protein, being the herein designated sIL-6R $\delta$ Val/L/IL-6 having a 13 amino acid peptide linker of sequence E-F-G-A-G-L-V-L-G-G-Q-F-M between the C-terminal Val-356 of sIL-6R and the N-terminal Pro-29 of IL-6R, said chimeric protein having the sequence set forth in Fig. 3 wherein the tripeptide of sequence E-F-M between positions 357-359 of Fig. 3 is replaced by said 13 amino acid peptide sequence.

15 (vii) A chimeric sIL-6R/IL-6 protein, wherein said protein is produced in mammalian cells in a fully processed form.

20 (viii) A chimeric sIL-6R/IL-6 protein, wherein said protein is produced in human cells.

(ix) A chimeric sIL-6R/IL-6 protein, wherein said protein is produced in CHO cells.

25 (x) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, as above, wherein said chimeric protein and analogs are characterized by being capable of inhibiting the growth of highly malignant cancer cells.

(xi) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, as above, wherein said chimeric protein and analogs are characterized by being capable of inhibiting the growth of highly malignant melanoma cells.

25 (xii) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, as above, wherein said chimeric protein and analogs are characterized by being

capable of eliciting the *in vivo* engraftment of human hematopoietic cells in bone marrow transplants.

(xiii) A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof, as above, wherein said chimeric protein and analogs are characterized by being capable of protecting liver against hepatotoxic agents.

The present invention also provides a DNA sequence encoding a chimeric sIL-6R/IL-6 protein and biologically active analogs thereof as noted above according to the invention.

In addition, the present invention also provides a DNA vector comprising a DNA sequence encoding a chimeric sIL-6R/IL-6 protein and biologically active analogs thereof of the invention, as noted above, said vector being suitable for expression of said chimeric protein in mammalian cells.

Embodiments of the DNA vector of the invention include :

(i) A DNA vector wherein said vector is suitable for expression of said chimeric protein in human cells.

(ii) A DNA vector wherein when said vector is expressed in mammalian or human cells, the expressed chimeric protein has a sequence that permits full processing of the chimeric protein by the mammalian or human cell and secretion of the fully processed chimeric protein from the cells into the culture medium in which said cells are grown.

(iii) A DNA vector, as above, wherein said vector is the herein designated plasmid pcDNAsIL-6R/IL-6 comprising a pcDNA3 vector containing the DNA sequence encoding the chimeric sIL-6R/IL-6 protein under the control of a cytomegalovirus (CMV) promoter.

(iv) A DNA vector, as above, wherein said vector is the herein designated plasmid pcDNA sIL-6R/L/IL-6 comprising a pcDNA3 vector containing the DNA sequence encoding the chimeric sIL-6R/IL-6 protein under the control of a

cytomegalovirus (CMV) promoter, and wherein in said DNA sequence encoding said chimeric sIL-6R/IL-6 protein there is inserted a linker sequence encoding a linker peptide at the EcoRI site placed between the sequence encoding the sIL-6R part and the sequence encoding the IL-6 part of the protein.

5        Likewise, the present invention also provides transformed mammalian cells containing a DNA vector as above, that is capable of expressing the sIL-6R/IL-6 chimeric protein sequence carried by said vector and of fully processing the expressed protein and secreting it into the culture medium in which said cells are grown.

10      An embodiment of these transformed cells are the herein described human embryonal kidney cells 293 (HEK293) transfected by the pcDNA sIL-6R/IL-6 vector, said cells being capable of expressing the sIL-6R/IL-6 chimeric protein, fully processing said protein and secreting said protein into the culture medium in which said cells are grown in the form of an about 85 kDa glycoprotein.

15      Another embodiment of transformed cells are the herein described CHO (Chinese Hamster Ovary) cells transfected by the pcDNA sIL-6R/IL-6 vector, said cells being capable of expressing the sIL-6-R/IL-6 chimeric protein, fully processing said protein into the culture medium in which said cells are grown in the form of an about 85 kDa glycoprotein.

20      The present invention also provides a method for producing a chimeric protein or biologically active analogs thereof, as above, comprising growing the aforesaid transformed cells under conditions suitable for expression, processing and secretion of said protein or analogs into the culture medium in which said cells are grown; and purifying said protein or analogs from said culture medium by  
25     immunoaffinity chromatography using monoclonal antibodies specific for sIL-6R.

The chimeric protein of the present invention has a number of uses including:

(i) use of a chimeric sIL-6R/IL-6 protein or analogs, salts of any one thereof, and mixtures thereof, as an inhibitor of cancer cells.

(ii) use, as in (i) above, as an inhibitor of highly malignant melanoma cells.

5 (iii) use of a chimeric sIL-6R/IL-6 protein or analogs, salts of any one thereof, and mixtures thereof, as an active ingredient for eliciting engraftment of human hematopoietic cells in bone marrow transplantation.

10 (iv) use of a chimeric sIL-6R/IL-6 protein or analogs, salts of any one thereof, and mixtures thereof, as an active ingredient for increasing hematopoiesis, for treating hepatic and neurological conditions, or for other applications in which IL-6 or sIL-6R are used.

Similarly, the chimeric protein of the present invention may be used to prepare medicaments for a number of medical indications, namely, a chimeric sIL-6R/IL-6 protein or analogs, salts of any one thereof and mixtures thereof, for use in the preparation of a medicament for treating cancers by way of inhibition of 15 cancer cells, or in the preparation of a medicament for enhancement of bone marrow transplantation by way of eliciting engraftment of human hematopoietic cells in bone marrow transplantation, or in the preparation of a medicament for increasing hematopoeisis, or in the preparation of a medicament for treating neurological disorders, or in the preparation of a medicament for other applications 20 in which IL-6 or sIL-6R are used.

Moreover, the present invention also provides a pharmaceutical composition comprising as active ingredient a chimeric sIL-6R/IL-6 protein or analog thereof as above, and a pharmaceutically acceptable carrier, diluent or excipient.

Embodiments of this pharmaceutical composition of the invention include :

25 (i) A pharmaceutical composition for the treatment of mammalian cancers.  
(ii) A pharmaceutical composition for the enhancement of bone marrow transplantation.

(iii) A pharmaceutical composition for the treatment of liver and neurological disorders, or for increasing hematopoiesis or for other applications in which IL-6 or sIL-6R are used.

The present invention also provides for a method for treating cancers in mammals, or for enhancing bone marrow transplantations, or for treating hepatic and neurological disorders, or for increasing hematopoiesis, or for other applications in which IL-6 or sIL-6R are used, comprising administering to a patient a pharmaceutical composition, as above, in a suitable dosage form and by a suitable route of administration.

In order to avoid doubt, the present invention relates to a chimera between IL-6 and sIL-6R in any order, i.e. the N-terminal and C-terminal portions may be reversed and the chimera is then an IL-6-sIL-6R protein, although it is referred to herein as sIL-6R/IL-6 protein throughout.

Other aspects and embodiments of the present invention are set forth or arise directly from the following detailed disclosure of the invention.

#### **Brief Description of the Drawings**

Figure 1 (A, B) depicts a schematic representation of the various vectors, reagents and process steps used in the construction of the chimeric DNA molecule encoding a chimeric protein in which is conserved the structure of the natural form of sIL-6R ending at the Val 356 residue followed by the sequence of the natural, mature, processed form of IL-6, as detailed in Example 1;

Figure 2 (A,B) shows the results obtained from the analysis performed to identify the sIL-6R $\delta$ Val/IL-6 p86 chimera by polyacrylamide gel electrophoresis (A) and by bioactivity profile (B), wherein in Fig. 2A there is shown a reproduction of a Coomassie stained gel on which were electrophoresed immunopurified

fractions eluted from affinity chromatography columns loaded with a secreted protein sample obtained from cell cultures transfected with a vector encoding the chimeric protein; and in Fig. 2B there is shown a graphic representation of the biological activity (growth inhibition of F10.9 melanoma cells) of each of the above noted fractions eluted from the affinity chromatography columns, all as detailed in Examples 2 and 3;

Figure 3 depicts the amino acid sequence (one-letter code) of the sIL-6R $\delta$ Val/IL-6 chimera in which is shown the different domains of the molecule, including the N-terminal signal peptide (line on top of sequence), the immunoglobulin-like (Ig-like) domain, the cytokine receptor N-domain (underlined), the cytokine C-domain (line on top of sequence) and the receptor pre-membrane region (the region between the C-domain and the transmembranal domain), all of the sIL-6R part of the chimera; as well as the mature IL-6 moiety (underlined below) of the chimera, as described in Examples 1 and 2;

Figure 4 (A, B) shows photographs of F10.9 melanoma cells in culture without (A) and with (B) treatment with the sIL-6R/IL-6 chimeric protein for 4 days, wherein in Fig. 4B there is apparent the morphological changes induced in such metastatic melanoma cells (F10.9 cells) by treatment with the sIL-6R/IL-6 chimera, as described in Example 3;

Figure 5 is a graphic representation of the results depicting the growth inhibition of F10.9 melanoma cells by the sIL-6R/IL-6 chimeric protein at various concentrations of the chimera ranging from about 0.12 ng/ml to about 150 ng/ml, where the chimera with only 3 amino acid linker IL-6RIL-6 as described in Example 3 is compared to a chimera with a long 13 amino acid linker (IL-6RLIL-6);

Figure 6 is a graphic representation of the results depicting the absence of growth-inhibitory effects on F10.9 melanoma cells of either isolated IL-6 alone

(dotted upper curve with open squares) at concentrations ranging from 0-40 ng/ml of IL-6 and sIL-6R alone (point of convergence of all curves on vertical axis where IL-6 concentration is zero); as well as the observed growth inhibitory effects when IL-6 and sIL-6R are added together at various concentrations of each wherein the  
5 IL-6 concentration ranges from 10 ng/ml to 40 ng/ml, and sIL-6R added at three concentrations of 100 ng/ml, 200 ng/ml and 400 ng/ml for each IL-6 concentration, as illustrated in the three lower curves (two dotted curves with open triangles and circles and full curve with closed squares), as described in Example 3;

Figure 7 is a reproduction of an autoradiogram of a Southern blot showing  
10 the requirement of the sIL-6R/IL-6 chimeric protein for successful engraftment of human hematopoietic stem cells during bone marrow transplantation in SCID-NOD mice (two right hand lanes representing the mice which received the sIL-6R/IL-6 chimeric protein in addition to the other necessary factors, SCF, FLT-3, and this in contrast to the three left hand lanes which represent mice having received only SCF  
15 and FLT-3 and SCF, FLT-3 as well as isolated, i.e. non-fused, IL-6 and sIL-6R), as described in Example 4;

Figure 8 is a Scatchard plot of the affinity characteristics of the sIL-6R/IL-6 chimera as compared to a mixture of IL-6 and sIL-6R, the values of the chimera depicted by filled squares and of the mixture by filled diamonds, the ratio of the  
20 slopes being 4 to 1;

Figure 9 shows the higher activity of the sIL-6R/IL-6 chimera on F10.9 melanoma cells as compared to the one of the mixture of sIL-6R + IL-6, or to the one of sIL-6R (without IL-6);

Figure 10 shows the sIL-6R/IL-6 chimera protection against liver toxicity,  
25 the values representing a mean of 4 experiments, filled squares representing IL-6-/ mice, filled diamonds representing IL-6-/ mice receiving IL-6, and filled stars representing IL-6-/ mice receiving the chimera;

Figure 11 depicts the amino acid sequence (one letter code) of the IL-6-sIL-6R $\delta$ Val chimera 3e, the linker being underlined; and

Figure 12 shows the biological activity on Fig. 9 melanoma cells of the chimera 3e (dark filled stars) compared to the sIL-6R/IL-6 chimera (filled squares) 5 and two mutants (Mutt 39 (HD) – filled diamonds) and Mutt NHD – light filled stars), as described in Example 9.

### Detailed description of the invention

The present invention concerns a chimeric sIL-6R/IL-6 protein and 10 biologically active analogs thereof which have essentially all of the naturally occurring forms of sIL-6R and essentially all of the naturally occurring forms of IL-6 fused together, the site of fusion of which may be by way of a linker peptide, as short as 3 amino acids, and which chimeric sIL-6R/IL-6 protein or analogs have a similar amount and pattern of glycosylation as that of naturally occurring sIL-6R 15 and IL-6. Such a chimeric sIL-6R/IL-6 protein produced in accordance with the present invention in mammalian cells, in particular, in human cells (see Examples 1-4 below) or CHO cells (see Example 6 below) was found to be efficiently expressed in such cells, to be highly glycosylated, and to have potent activity on tumor cells which show no response at all to IL-6 or sIL-6R alone.

20 More particularly, in accordance with the present invention it has been observed (see Examples 1-3 below) that the aforesaid chimeric sIL-6R/IL-6 protein of the invention causes growth arrest of highly malignant mammalian cells such as the F10.9 melanoma cells at concentrations lower than needed when a mixture of non-fused sIL-6R and IL-6 is used. This is a particularly significant result in view 25 of the fact that such F10.9 melanoma cells continue to grow normally when treated with only IL-6 or only sIL-6R separately, and undergo growth arrest only when exposed to relatively high dosages of a combination of non-fused IL-6 and sIL-6R.

Accordingly, the chimeric sIL-6R/IL-6 protein of the present invention is surprisingly a more potent inhibitor of these highly malignant melanoma cells than a mixture of its separate parts, i.e. a mixture of non-fused IL-6 and sIL-6R. The chimeric protein of the present invention is thus particularly useful as an active 5 ingredient for treating various kinds of cancers.

The higher activity of the chimeric sIL-6R/IL-6 protein is accounted for by its higher affinity for gp130 than that of the mixture of non-fused IL-6 and sIL-6R (Example 7).

Furthermore, it has also been found in accordance with the present invention 10 (see Example 4 below) that a chimeric sIL-6R/IL-6 molecule of the present invention is particularly useful for enhancing bone marrow transplantation. In fact, using a known protocol for engraftment of human bone marrow cells into severe combined immunodeficient (SCID) mice, in which stem-cell factor (SCF) and Flt3-ligand are used for enabling survival and proliferation of the most primitive 15 pluripotential hematopoietic stem cells capable of long-term engraftment into recipient bone marrow, it was found that these two factors, SCF and Flt3-ligand, were insufficient to promote the engraftment of human cells into the recipient mouse bone marrow, and that only when the chimeric sIL-6R/IL-6 protein was also added was engraftment successful. This finding indicates that the chimeric protein 20 may be essential in such engraftment protocols. In the same experiments, non-fused IL-6 and sIL-6R when added separately, were insufficient to promote successful bone marrow transplantation and when added together were much less active than the chimeric sIL-6R/IL-6 protein, i.e. at an effective concentration of 100 ng/ml the sIL-6R/IL-6 chimeric protein promoted successful bone marrow transplantation, 25 while the two separate non-fused sIL-6R and IL-6 when added together at even higher concentrations (sIL-6R from 125-1250 ng/ml, IL-6 from 50-200 ng/ml), were much less active in promoting such transplantation.

The above chimeric sIL-6R/IL-6 protein of the invention is preferably a recombinant glycosylated sIL-6R/IL-6 chimera produced in human cells or in any other suitable mammalian cell expression system such as hamster CHO cells which is capable of glycosylating proteins as do human cells and which introduces the same post-translational modifications as do human cells. An important characteristic is that the chimeric glycoprotein so-produced is processed and modified as are the natural sIL-6R and IL-6 parent molecules found in the human body, without truncation and without addition of extraneous unnatural polypeptide sequences, with the exception of the very short tripeptide or when a longer linker peptide is incorporated between the sIL-6R and IL-6 moieties of the chimeric protein.

To prepare the above preferred chimeric protein of the invention, the following features of the naturally-occurring sIL-6R and IL-6 were considered : It is known that the IL-6R present in human cell membranes is produced by a cDNA encoding 468 aminoacids comprising a signal peptide, an Immunoglobulin (Ig) like domain, a cytokine binding domain, a transmembrane region and a cytoplasmic domain (Yamasaki et al, 1988). A soluble form of sIL-6R is found in body fluids which has, like the mature IL-6R from membranes, an N-terminus corresponding to Leu-20 (Novick et al, 1990) and a C-terminus corresponding to Val-356 just before the transmembrane region of IL-6R (see co-owned U.S. Pat. No. 5,216,128 and EP 413.908 B1). In order to fuse this sIL-6R sequence to IL-6, an EcoRI restriction site was introduced following Val-356. The sequence of the mature IL-6 starting at Pro-29 of the IL-6 cDNA and ending at Met-212 (Zilberstein et al, 1986; Hirano et al, 1986) was introduced after this EcoRI site. At this EcoRI site there could also, but not necessarily, be introduced a linker peptide of desired length to distance the sIL-6R and IL-6 moieties from each other in the chimeric protein. As set forth in the Examples below, two different chimeric proteins were produced as examples of

such possible chimeric proteins, one having a tripeptide linker and the other having a 13-amino acid residue linker at this EcoRI site, both being essentially equally active biologically.

The present invention also concerns analogs of the above chimeric sIL-6R/IL-6 protein of the invention, which analogs retain essentially the same biological activity of the chimeric protein having essentially only the naturally occurring sequences of sIL-6R and IL-6. Such analogs may be ones in which up to about 30 amino acid residues may be deleted, added or substituted by others in the sIL-6R and/or IL-6 moieties of the chimeric protein, such that modifications of this kind do not substantially change the biological activity of the chimeric protein analog with respect to the chimeric protein itself and in which the sIL-6R moiety of such analogs essentially retains the naturally occurring structure (before processing - see Fig. 3) of a signal peptide, Ig-like domain, cytokine receptor N-domain, cytokine receptor C-domain, and receptor pre-membrane domain. Likewise, such chimeric protein analogs should retain essentially the naturally-occurring mature form of the IL-6 moiety. The various analogs may differ most from each other and from the basic chimeric protein molecule (that with essentially only naturally-occurring sIL-6R and IL-6 sequences) at the site of the linker peptide which joins the sIL-6R and IL-6 moieties in the chimeric protein. Such a linker may be up to about 30 amino acids in length, and serves to separate the sIL-6R and IL-6 moieties from each other in the chimeric protein. As regards such a linker, care should be taken to choose its sequence (and hence also to test biologically in appropriate standard assays each such analog) such that it will, for example, not result in incorrect folding of the chimeric protein which may render it inactive, or it will not result in rendering the chimeric protein analog an immunogenic protein which will elicit antibodies against it in a patient to be treated therewith with the

DRAFT - 10/10/2002

result that such an analog will be ineffective at least as a medium- or long- term medicament.

As regards the above analogs of the chimeric protein of the invention, these analogs are those in which one or more and up to about 30 of the amino acid residues of the basic chimeric protein of the invention are replaced by different amino acid residues, or are deleted, or one or more amino acid residues are added to the original sequence of chimeric protein of the invention (that with essentially only the naturally-occurring sIL-6R and IL-6 sequences) without changing considerably the activity of the resulting products as compared with the basic chimeric protein of the invention. These analogs are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable therefor.

Any such analog preferably has a sequence of amino acids sufficiently duplicative of that of the basic sIL-6R/IL-6 chimera such as to have substantially similar activity thereto. Thus, it can be determined whether any given analog has substantially the same activity as the basic chimeric protein of the invention by means of routine experimentation comprising subjecting such an analog to the biological activity tests set forth in Examples 2-4 below.

Analogs of the chimeric protein which can be used in accordance with the present invention, or nucleic acids coding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. et al., *Principles of Protein Structure*, Springer-Verlag, New York, 1978; and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a

presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel et al, *supra*, at §§ A.1.1-A.1.24, and Sambrook et al, Current Protocols in Molecular Biology, Interscience N.Y. §§6.3 and 6.4 (1987, 1992), at Appendices C and D.

5 Preferred changes for analogs in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions of those in the chimeric protein having essentially the naturally-occurring sIL-6R and IL-6 sequences, may include synonymous amino acids within a group which have sufficiently similar physicochemical properties  
10 that substitution between members of the group will preserve the biological function of the molecule, Grantham, Science, Vol. 185, pp. 862-864 (1974). It is clear that insertions and deletions of amino acids may also be made in the above-defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under thirty, and  
15 preferably under ten, and do not remove or displace amino acids which are critical to a functional conformation, e.g., cysteine residues, Anfinsen, "Principles That Govern The Folding of Protein Chains", Science, Vol. 181, pp. 223-230 (1973). Analogs produced by such deletions and/or insertions come within the purview of the present invention.

20 Preferably, the synonymous amino acid groups are those defined in Table I. More preferably, the synonymous amino acid groups are those defined in Table II; and most preferably the synonymous amino acid groups are those defined in Table III.

**TABLE I** Preferred Groups of Synonymous Amino Acids

	Amino Acid	Synonymous Group
5	Ser	Ser, Thr, Gly, Asn
	Arg	Arg, Gln, Lys, Glu, His
	Leu	Ile, Phe, Tyr, Met, Val, Leu
	Pro	Gly, Ala, Thr, Pro
	Thr	Pro, Ser, Ala, Gly, His, Gln, Thr
	Ala	Gly, Thr, Pro, Ala
10	Val	Met, Tyr, Phe, Ile, Leu, Val
	Gly	Ala, Thr, Pro, Ser, Gly
	Ile	Met, Tyr, Phe, Val, Leu, Ile
	Phe	Trp, Met, Tyr, Ile, Val, Leu, Phe
	Tyr	Trp, Met, Phe, Ile, Val, Leu, Tyr
15	Cys	Ser, Thr, Cys
	His	Glu, Lys, Gln, Thr, Arg, His
	Gln	Glu, Lys, Asn, His, Thr, Arg, Gln
	Asn	Gln, Asp, Ser, Asn
	Lys	Glu, Gln, His, Arg, Lys
20	Asp	Glu, Asn, Asp
	Glu	Asp, Lys, Asn, Gln, His, Arg, Glu
	Met	Phe, Ile, Val, Leu, Met
	Trp	Trp

094-000000000000

**TABLE II** More Preferred Groups of Synonymous Amino Acids

	Amino Acid	Synonymous Group
5	Ser	Ser
	Arg	His, Lys, Arg
	Leu	Leu, Ile, Phe, Met
	Pro	Ala, Pro
	Thr	Thr
	Ala	Pro, Ala
10	Val	Val, Met, Ile
	Gly	Gly
	Ile	Ile, Met, Phe, Val, Leu
	Phe	Met, Tyr, Ile, Leu, Phe
	Tyr	Phe, Tyr
15	Cys	Cys, Ser
	His	His, Gln, Arg
	Gln	Glu, Gln, His
	Asn	Asp, Asn
	Lys	Lys, Arg
20	Asp	Asp, Asn
	Glu	Glu, Gln
	Met	Met, Phe, Ile, Val, Leu
	Trp	Trp

**TABLE III** Most Preferred Groups of Synonymous Amino Acids

	<b>Amino Acid</b>	<b>Synonymous Group</b>
	Ser	Ser
	Arg	Arg
5	Leu	Leu, Ile, Met
	Pro	Pro
	Thr	Thr
	Ala	Ala
	Val	Val
10	Gly	Gly
	Ile	Ile, Met, Leu
	Phe	Phe
	Tyr	Tyr
	Cys	Cys, Ser
15	His	His
	Gln	Gln
	Asn	Asn
	Lys	Lys
	Asp	Asp
20	Glu	Glu
	Met	Met, Ile, Leu
	Trp	Met

25 Examples of production of amino acid substitutions in proteins which can be used for obtaining analogs of the chimeric protein for use in the present invention include any known method steps, such as presented in US patents RE

33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Koths et al., 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and lysine substituted proteins presented in US patent No. 4,904,584 (Shaw et al).

5        In another preferred embodiment of the present invention, any analog of the chimeric protein for use in the present invention has an amino acid sequence essentially corresponding to that of the above noted basic chimeric protein of the invention. The term "essentially corresponding to" is intended to comprehend analogs with minor changes to the sequence of the basic chimeric protein which  
10 do not affect the basic characteristics thereof, particularly insofar as its ability to inhibit cancer cell proliferation or promote bone marrow transplantations, for example, is concerned. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding the  
15 chimeric protein of the invention, resulting in a few minor modifications, and screening for the desired activity in the manner discussed above.

Analogs in accordance with the present invention include those encoded by a nucleic acid, such as DNA or RNA, which hybridizes to DNA or RNA under stringent conditions and which encodes a chimeric protein in accordance  
20 with the present invention, comprising essentially all of the naturally-occurring sequences encoding sIL-6R and IL-6. For example, such a hybridizing DNA or RNA may be one encoding the same protein of the invention having, for example, the sequence set forth in Fig. 3, but which differs in its nucleotide sequence from the naturally-derived nucleotide sequence by virtue of the degeneracy of the genetic code, i.e., a somewhat different nucleic acid sequence  
25 may still code for the same amino acid sequence, due to this degeneracy. Further, as also noted above, the amount of amino acid changes (deletions, additions,

substitutions) is limited to up to about 30 amino acids, such that even with the maximum amount of changes, analogs in accordance with the present invention will be those which essentially retain the leader sequence (before processing), Ig-like domain, cytokine receptor N- and C- domains and receptor pre-membrane region (the region between the C-domain and the transmembranal domain) in the sIL-6R moiety and essentially all of the IL-6 moiety. Such nucleic acid would be a prime candidate to determine whether it encodes a polypeptide which retains the functional activity of the chimeric protein of the present invention. The term "stringent conditions" refers to hybridization and subsequent washing conditions which those of ordinary skill in the art conventionally refer to as "stringent". See Ausubel et al., Current Protocols in Molecular Biology, supra, Interscience, N.Y., para. 6.3 and 6.4 (1987, 1992), and Sambrook et al., supra. Without limitation, examples of stringent conditions include washing conditions 12-20°C below the calculated Tm of the hybrid under study in, e.g. 2 x SSC and 0.5% SDS for 5 minutes, 2 x SSC and 0.1% SDS for 15 minutes; 0.1 x SSC and 0.5% SDS at 37°C for 30-60 minutes and then a 0.1 x SSC and 0.5% SDS at 68°C for 30-60 minutes. Those of ordinary skill in this art understand that stringency conditions also depend on the length of the DNA sequences, oligonucleotide probes (such as 10-40 bases) or mixed oligonucleotide probes. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC. See Ausubel, supra.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the chimeric protein of the invention or analogs thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and

the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar activity to the chimeric protein of the invention or its  
5 analogs.

The present invention also concerns DNA sequences encoding the above chimeric protein of the invention and its analogs, as well as DNA vectors carrying such DNA sequences for expression in suitable mammalian, preferably human, cells. An embodiment of a vector of the invention is a plasmid pcDNA sIL-6R/IL-6 comprising the pcDNA3 vector (Invitrogen) containing the sIL-6R/IL-6 fused sequences under the control of a cytomegalovirus (CMV) promoter.  
10

The present invention also concerns transformed mammalian, preferably human, cells capable of expressing the above proteins of the present invention. An embodiment of such transformed cells are human embryonal kidney cells 293 (HEK 15 293, ATCC CRL 1573) transfected by pcDNA sIL-6R/IL-6 which secrete the fused sIL-6R/IL-6 chimeric as a 85 kDa glycoprotein.

A further embodiment is plasmid pcDNA sIL-6R/L/IL-6 which differs from the above pcDNA sIL-6R/IL-6 by insertion in the EcoRI site of short linkers encoding 10 additional aminoacids. A number of different sequences, of various 20 lengths, can be introduced to optimize the distance between sIL-6R and IL-6.

The invention also includes a chimeric protein in which the IL-6 moiety precedes the sIL-6R (as in Fig. 11).

The present invention further concerns a method for producing and purifying the chimeric protein of the invention or its analogs which comprises growing the 25 above transformed cells under conditions suitable for expression and secretion of the chimeric protein product into the culture medium and then purifying the

secreted protein by immunoaffinity chromatography using anti-sIL-6R monoclonal antibodies 34.4 as noted in Example 2 and 5 below.

The invention also concerns a pharmaceutical composition comprising as active ingredient an sIL-6R/IL-6 chimera or analogs thereof or mixtures thereof or salts thereof and a pharmaceutically acceptable carrier, diluent or excipient. An embodiment of the pharmaceutical composition of the invention includes a pharmaceutical composition for enhanced IL-6 type action, for the treatment of cancers, for bone marrow transplantation, for increase of hematopoiesis, in particular thrombopoiesis, for treatment of neurological conditions, for the treatment of liver disorders, and other applications of IL-6 or related cytokines.

The pharmaceutical compositions of the invention are prepared for administration by mixing the chimeric protein, or its analogs with physiologically acceptable carriers, and/or stabilizers and/or excipients, and prepared in dosage form, e.g., by lyophilization in dosage vials. The method of administration can be via any of the accepted modes of administration for similar agents and will depend on the condition to be treated, e.g., intravenously, intramuscularly, subcutaneously, by local injection or topical application, or continuously by infusion, etc. The amount of active compound to be administered will depend on the route of administration, the disease to be treated and the condition of the patient. Local injection, for instance, will require a lower amount of the protein on a body weight basis than will intravenous infusion.

The present invention also concerns uses of the chimeric protein of the invention or its analogs or mixtures thereof for the treatment of cancers, for bone marrow transplantations, for increasing hematopoiesis, especially thrombopoiesis, for treatment of neurological conditions, for protection of liver tissues in patients with necrotic diseases due to chemicals (e.g. carbon tetrachloride, alcohol, paracetamol) or other causes (e.g. viral, surgical) and for

use in other applications of IL-6 or related cytokines. Likewise, the present invention also concerns the chimeric protein or analogs thereof or mixtures thereof for use in the preparation of medicaments for treating the above-mentioned ailments or for use in the above noted indications.

5 In addition to the above mentioned methods of treatment, also ex-vivo procedures and gene therapy with the chimera and or DNA encoding it are contemplated.

The present invention will now be described in more detail in the following non-limiting Examples and the accompanying drawings.

10

**Example 1: Construction of the sIL-6RδVal/IL-6 chimera expression vector**

In Figure 1, there is shown a schematic flow-diagram of the steps taken to construct the expression vector carrying the sequence coding for the sIL-6RδVal/IL-6 chimeric protein, inclusive of all the various starting and intermediary vectors, various reagents and reaction steps. This construction procedure was essentially using techniques well known in the art for constructing expression vectors of choice (see, for example, Sambrook et al., 1989). The procedure was, briefly, as follows :

A library of cDNAs from human breast carcinoma T47D cells was cloned in  
20 the lamda ( $\lambda$ ) gt11 bacteriophage and screened with oligonucleotide probes derived from the IL-6R sequence of Yamasaki et al (1988). One  $\lambda$ gt11 cDNA clone was isolated which had the entire human IL-6R coding sequence. The insert was excised from  $\lambda$ gt11 by EcoRI and cloned in the Multiple Cloning Site (MCS) of the E.coli phagemid Blue Script pBS/SK (Stratagene Cloning Systems, LaJolla, California).  
25 This plasmid pBS/SK-IL-6R ( Figure 1) was cut by EcoRI which was then blunt-ended and recut with EcoRV to isolate the 5' fragment of IL-6R of 959 base pairs (bp) ending at the EcoRV site of IL-6R (coordinate 1203). This fragment

094376 - 047000

extracted from an agarose gel electrophoresis was cloned in a new pBS/SK vector opened at the EcoRV of the MCS (pBS/SK-sIL-6R-RV in Figure 1).

The above noted, previously obtained pBS/SK-IL-6R DNA was subjected to Polymerase Chain Reaction (PCR) to amplify a 368 bp fragment between the forward primer 1137-1156 and the reverse primer 1505-1488. The reverse primer was synthesized with an EcoRI site immediately following the codon for Valine-356 of the IL-6R (see Figure 1), since this Valine residue was previously determined to be the carboxy-terminal amino acid of the natural form of the soluble SIL-6R excreted in human urine (Novick et al, 1990; Oh et al, 1996; co-owned U.S. Pat. No. 5,216,128 and EP Pat. No. EP 413908 B1). The PCR product was cut by EcoRV and by EcoRI and ligated into pBS/SK-sIL-6R-RV between the EcoRV site of IL-6R and the EcoRI site of the MCS (Figure 1). The resulting plasmid pBS-sIL-6R- $\delta$ Val-RI was then shortened to remove 5' untranslated sequences by ligation of the HindIII site of MCS with the NcoI site at base pair 410 of IL-6R (both sites being first blunt-ended), to yield pBS-sIL-6R- $\delta$ Val-RI-NcoI (Figure 1).

The IL-6 sequence was derived from plasmid pKK $\beta$ 2-7 which, as previously described (Chen et al, 1988), was constructed by insertion of the BstNI-cut IFN- $\beta$ 2/IL-6 cDNA (Zilberstein et al, 1986) into the EcoRI site of the E.coli expression vector pKK223-3 (Pharmacia, Uppsala, Sweden) using a synthetic oligonucleotide with an EcoRI site followed by a Methionine codon and the codon for Proline-29 of IL-6 and ending at a BstNI (EcoRII) site. The IL-6 cDNA insert of pKK $\beta$ 2-7 ends 7 base pairs after the termination codon in a NlaIV site and is followed 11 bp later by the HindIII site of the pKK223-3 vector (Figure 1). The pKK $\beta$ 2-7 DNA was cut with HindIII, blunt-ended and recut with EcoRI and the IL-6 cDNA inserted into pBS-sIL-6R- $\delta$ Val-RI-NcoI so as to fuse the mature sequence of IL-6 (starting at Proline-29) immediately after Valine-356 of the IL-6R and separated by only 3 codons (Glu-Phe-Met). The resulting plasmid

pBS/SK-sIL-6R/IL-6 (Figure 1) was then recut at the SalI and NotI sites of its MCS and the insert was cloned into the EcoRV site of pcDNA3 (Invitrogen Corporation, San Diego, California). The resulting plasmid pCDNA3-sIL-6R/IL-6 (Figure 1) contains the insert downstream of the strong cytomegalovirus (CMV) promoter and followed by a polyadenylation site insuring efficient transcription of the sIL-6RδVal/IL-6 chimera. The conservation of the 5'end of the sIL-6R in the chimera ensures that upon expression in mammalian cells the signal peptide function and processing of the N-terminus of the chimeric protein will be as in the natural sIL-6R.

As indicated above, an advantageous characteristic of the sIL-6RδVal/IL-6 construct is that it is essentially the fusion of the natural form of sIL-6R and of the natural form of IL-6 as they exist in the human body, and without extraneous polypeptide sequences. However, the conservation of the EcoRI site in the sIL-6RδVal/IL-6 construct (Figure 1) allows to easily introduce linker polypeptide segments between the sIL-6R and the IL-6 moieties. One such construct with the 13-amino acid linker sequence Glu-Phe-Gly-Ala-Gly-Leu-Val-Leu-Gly-Gly-Gln-Phe-Met introduced between Val-356 of sIL-6R and Pro-29 of IL-6, was also constructed (sIL-6RδVal/L/IL-6).

**Example 2: Expression of the sIL-6RδVal/IL-6 chimera in human cells.**

Using essentially standard techniques of mammalian cell culture, cell transfection and analysis of the transfected cells for expression of the newly introduced DNA sequence to be expressed (for procedures, see, for example, Sambrook et al., 1989), the above plasmid construct (Example 1) was used to transfet human cells and its expression therein was assessed. Briefly, the following procedures were employed :

Human HEK 293 cells (ATCC CRL 1573, transformed primary human embryonal kidney cells) were transfected with the plasmic construct pCDNA3-sIL-6R/IL-6 DNA (set forth in Example 1 above). Log phase cultures of HEK 293 were trypsinized and seeded in 9cm Nunc plates ( $2.5 \times 10^6$  cells/plate).

5 One day later, transfection was carried out with 10 µg pCDNA3-sIL-6R/IL-6 DNA by the CaPO<sub>4</sub> precipitation procedure (Sambrook et al, 1989) and 1 hour later the medium changed to DMEM-10% FCS and the culture continued for an additional 16 hours. After changing the medium to DMEM-2% FCS, the secreted proteins were collected for two consecutive periods of 48 hours. Debris was removed by  
10 centrifugation at 1,000 rpm for 10 minutes and the supernatant tested by an ELISA for sIL-6R using polyclonal rabbit anti-sIL-6R and mouse McAB 17.6 (Novick et al, 1991). A concentration of 1.2 µg/ml sIL-6R -equivalents was found, indicative of very efficient expression of the chimeric sIL-6R/IL-6 protein in the transfected human cells.

15 Immunopurification of the secreted chimeric protein (sIL-6R/IL-6) was carried out with Monoclonal Antibody 34.4 specific to an epitope in the extracellular domain of human sIL-6R (Novick et al, 1991; Halimi et al, 1995). The 34.4 hybridoma cells were grown in the peritoneal cavity of mice and the immunoglobulin (Ig) fraction was obtained from the ascitis fluid by ammonium  
20 sulfate precipitation. Affigel-10 (Bio-Rad Labs, Richmond, California) was used to immobilize McAB 34.4 (15 mg Ig coupled to 1 ml Affigel-10). The supernatants containing the secreted proteins from the HEK 293 cells transfected by pCDNA3-sIL-6R/IL-6 were adsorbed on columns of McAB 34.4 (0.3 ml column for 15 ml supernatant). After washing with PBS, the bound proteins were eluted by  
25 25 mM citric acid pH 2.5, then immediately neutralized by 1 M Hepes buffer pH 8.5 and dialyzed overnight (about 8-12 hrs) against PBS.

Analysis of the immunopurified protein by polyacrylamide gel electrophoresis in SDS showed a unique protein band stained by Coomassie blue (Figure 2). The molecular weight of the protein was 85 kilodaltons as expected from the fusion of the glycosylated forms of sIL-6R $\delta$ Val (60 kDa as shown in Oh et al, 1996) and glycosylated IL-6 (23-26 kDa as shown in Zilberstein et al, 1986). The aminoacid sequence of the sIL-6R/IL-6 is 543 aminoacids, which after processing of the signal peptides would predict a protein of 524 aminoacids or about 58 kDa (Figure 3). The much larger size of the sIL-6R/IL-6 chimera produced from the recombinant DNA in human cells indicates that glycosylation accounts for a sizable portion of the molecule.

**Example 3: The sIL-6R/IL-6 chimera arrests growth and induces differentiation of metastatic melanoma cells.**

The F10.9 clone derived from B16 melanoma cells forms highly metastatic tumors in C57Black/6 mice which kill the animals from pulmonary metastases within 2-3 months (Katz et al, 1995). Addition of the sIL-6R/IL-6 chimeric protein to F10.9 cells culture produces a profound morphological change in the cells and an arrest in their growth (Figure 4). The F10.9 cells treated by the chimera become elongated, with protruding dendritic extensions, resembling the spindloid differentiation of embryonic melanocytes or glial cells.

The growth of the cells was quantitated 4 days after seeding  $3 \times 10^3$  cells in wells of a 96-well microplate in 0.2 ml RPMI 1640 medium with 10% FCS. The cells were fixed in 12.5% glutaraldehyde for 30 minutes, washed in water and stained with 0.1% crystal violet for 30 minutes. After thorough washing and drying, the stain was extracted by 10% acetic acid and the optical density determined at 540 nm. The chimera produced a dose-dependent inhibition of growth with a complete growth inhibition at concentrations as low as 10 ng/ml of the chimeric (p85) protein

(Figure 5). Both chimeric proteins sIL-6R $\delta$ Val/IL-6 and sIL-6R $\delta$ Val/L/IL-6 (chimera with the longer linker between the sIL-6R and IL-6 moieties, see Example 1) were similarly active. This result also serves to show that the linker peptide between the sIL-6R and IL-6 moieties in the chimera, is not essential for the activity of the chimera as the above sIL-6R $\delta$ Val/IL-6 chimera has only a very short 3 amino acid linker while the above sIL-6R $\delta$ Val/L/IL-6 has a longer 13 amino acid linker peptide, but both have essentially the same activity in inhibiting the growth of the metastatic cells. In contrast, neither IL-6 alone, nor the sIL-6R  $\delta$ Val alone inhibit the growth of these melanoma cells (Figure 6) demonstrating the unique activity of the sIL-6R/IL-6 (p85) chimeric protein. To obtain a similar effect, a mixture of 200-400 ng/ml IL-6 and 125 ng/ml sIL-6R $\delta$ Val is required (Figure 6). When calculated in molar concentrations, the maximal inhibition of F10.9 cells required 7.5 nM IL-6 and 2 nM sIL-6R $\delta$ Val versus only 0.12 nM of the sIL-6R/IL-6 chimera.

The growth inhibitory activity of the p85 sIL-6R/IL-6 chimeric protein was followed during the immunopurification on McAB 34.4 columns (see Example 2). The pattern of activity corresponded to the intensity of the p85 band seen in the different fractions of the SDS polyacrylamide gel electrophoresis in Figure 2.

**Example 4: The sIL-6R/IL-6 Chimera is essential for engraftment of human bone marrow transplanted cells**

Engraftment of hematopoietic stem cells from human bone marrow can be studied after transplantation into severe combined immunodeficient (SCID) mice (Vormoor et al, 1994). SCID-NOD mice were subjected to sublethal irradiation and injected in the tail vein with  $3 \times 10^5$  human CD34 $^+$  bone marrow cells. Prior to injection, the purified CD34 $^+$  cells were maintained for 3 days in liquid cultures with different combinations of cytokines. After one month, the mice were sacrificed

and bones were taken to collect the bone marrow cells. The engraftment of human cells in the SCID-NOD recipient mice was evaluated by Southern blot hybridization to human repetitive DNA.

Stem-cell factor (SCF, steel factor or ckit-ligand) and Flt3-ligand (flt3/flk2 tyrosine kinase receptor ligand) have been found important for survival and proliferation of the most primitive pluripotential hematopoietic stem cells capable of long-term engraftment in recipient bone marrow (McKenna et al, 1995). As seen in Figure 7, these two factors by themselves were insufficient to promote the engraftment of human cells in the bone marrow of the SCID-NOD recipient mice.

10 Addition of the sIL-6R/IL-6 chimeric protein was required for engraftment to be detected at significant levels. At 100 ng/ml, the sIL-6R/IL-6 chimera was much more active than the isolated IL-6 (50-200 ng/ml) and sIL-6R (125-1250 ng/ml) (Figure 7). The requirement for the sIL-6R/IL-6 chimera indicates that this protein is essential for the survival and proliferation of the non-committed pluripotential 15 hematopoietic stem cells which can home into and repopulate the bone marrow environment, indicating that this protein may be useful in bone marrow transplantation clinical protocols.

This is the first demonstration that the sIL-6R/IL-6 chimera has the following at least two newly found activities :

- 20 (i) When added together with both of the factors SCF and Flt3- ligand to human hematopoietic primitive progenital cells, it promotes their survival and proliferation; and
- (ii) It is active (and apparently essential) in an *in vivo* model of a human bone marrow transplantation in immuno-deficient mice.

**Example 5 : The sIL-6R/IL-6 chimera is active on highly purified primitive hematopoietic stem cells**

Human cord blood mononuclear cells were subjected to fractionation of low density mononuclear cells (NMC) on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) followed by a mini MACS kit (Miltney Biotec, Bergisch Gladbach, Germany) to prepare a 80% pure population of CD34<sup>+</sup> cells. These cells were then passed over immobilized anti-CD38 monoclonal antibody or sorted by fluorescence activated cell sorting and the CD34<sup>+</sup>CD38<sup>-</sup> population, corresponding to about 0.1% of the original cells, was recovered. These purified stem cells (20,000 cells) were placed in suspension cultures in 0.5 ml RPMI medium, 10% fetal calf serum (FCS), 1% bovine serum albumin containing 50 ng/ml stem cell factor (SCF) and 100 ng/ml flt3-ligand (FL) (both from R&D Systems, Minneapolis, MN). Half of the cultures were supplemented with 100 ng/ml sIL-6R/IL-6 chimera, the others were cultured without. Incubation was at 37°C in 5% CO<sub>2</sub>, for 6 days. The number of bone marrow repopulating cells was evaluated by injection (i.v.) of all the cells from these *in vitro* cultures into sub-lethally irradiated NOD-SCID mouse. The mice were maintained in germ-free conditions. After 6 weeks, the mice were sacrificed and the marrow of their long bones was recovered. These bone marrow (BM) cells were plated on semi-solid 0.9% methylcellulose plates with 30% FCS, 50 µM β-mercaptoethanol, 50 ng/ml SCF, 5 ng/ml IL-3, 5 ng/ml GM-CSF, 6 u/ml erythropoietin (all R&D Systems). The cultures contained also human serum, conditions which prevent growth of mouse colonies. The results (Table IV) indicated that the sIL-6R/IL-6 chimera addition to the suspension cultures produces a 30-50 fold increase in the number of human colony forming cells (CFU) recovered from the transplanted mice as compared to SCF and FL alone. This represents a large increase in the number of SCID-repopulating stem cells present in the suspension cultures at day 6 compared to day 0. In the absence of sIL-6R/IL-6

chimera, SCF and FL produced no increase in the number of stem cells during the 6 days of suspension culture. The DNA of the BM cells recovered from the transplanted NOD/SCID mice was analyzed by Southern blot as in Example 4. The amount of human DNA recovered was 10 times higher when the mice received the 5 cells cultured with chimera as compared to without chimera.

The CFU progenitors from bone marrow of NOD/SCID mice as in Table IV, gave rise to hematopoietic cells of different myeloid lineages (macrophage and granulocyte) as well as erythroid and lymphoid lineages (e.g. CD19<sup>+</sup>, CD56<sup>+</sup>) only when the human blood cells had been cultured with sIL-6R/IL-6 chimera prior to 10 transplantation.

**TABLE IV Human stem cells capable of repopulating bone marrow of NOD/SCID mice**

15	Additions during the suspension culture of CD34 <sup>+</sup> CD38 <sup>-</sup> human cells from Cord Blood	Days of culture	Number of human hematopoietic colonies formed from BM of transplanted NOD/SCID mice
20	SCF + FL	0	4
	SCF + FL + sIL-6R/IL-6	6	2-3
		6	50-100

25 Additional experiments compared the effect of sIL-6R/IL-6 on the cord blood CD34<sup>+</sup>CD38<sup>+</sup> population to those on the highly purified CD34<sup>+</sup>CD38<sup>-</sup> stem cells. The *in vitro* expansion of the highly purified cells was much more strongly

enhanced by sIL-6R/IL-6 than that of the less purified cells (Table V). This indicates that the most primitive stem cells are the preferential target of the sIL-6R/IL-6 effect on cell expansion.

5

**TABLE V** *In vitro* Expansion of Hematopoietic Stem Cells

Cell population seeded (20,000 cells)	Cell number at day 6 with SC + FL	Cell number at day 6 with SC + FL + sIL-6R/IL-6
--	--------------------------------------	--

10

Expt. 1

CD34 <sup>+</sup> CD38 <sup>+</sup>	780,000	675,000 (x 0.86)
CD34 <sup>+</sup> CD38 <sup>-</sup>	42,000	153,000 (x 3.6)

15

Expt. 2

CD34 <sup>+</sup> CD38 <sup>+</sup>	330,000	507,000 (x 1.5)
CD34 <sup>+</sup> CD38 <sup>-</sup>	3,000	18,000 (x 6.0)

20

The *in vitro* maintenance of the Bone Marrow-repopulating activity was measured by increasing the length of the suspension of cultures of highly purified CD34<sup>+</sup>CD38<sup>-</sup> stem cells before injection to the NOD/SCID mice. The engraftment was evaluated by the proportion of human DNA in the Bone Marrow of the recipient mice 6 weeks after i.v. injection of the cultured cells. When sIL-6R/IL-6 was added to SCF and FL during the cultures, a high engraftment (> 1% human DNA) was still observed after two weeks of culture, and the engraftment was higher than in the non-cultured cells. In contrast thereto, experiments with cultures

25

COLLECTIVE  
COPYRIGHT  
BY THE AUTHOR(S)

containing SCF, FL, GM-CSF, IL-3 have shown that no SCID-repopulating cells remain after one week of culture (Bhata, M. et al., J. Exp. Med. 186, 619-624, 1997).

These results show that sIL-6R/IL-6 allows to expand and maintain, human primitive stem cells capable of engraftment in recipient bone marrow. The stem cells remain active in a non-differentiated state while multiplying. The sIL-6R/IL-6 chimera provides a new means to culture engrafting hematopoietic cells. This may also allow to use retroviral vectors to introduce genes into engrafting stem cells, in protocols of gene therapy. Until now, this has not been possible with human stem cells because these primitive cells could not be maintained *in vitro* in a cycling state, as required for retroviral DNA integration. The sIL-6R/IL-6 chimera solves this problem.

#### Example 6 : Production of IL-6R/IL-6 chimera in CHO cells

DNA of plasmid sIL-6R/IL-6 pcDNA3 as in Figure 1, was co-transfected into Chinese Hamster Ovary (CHO) cells, together with DNA of plasmid pDHFR as described in Mory et al (DNA 5, 181-193, 1986). Among the transfectants growing in 50 nM Methotrexate, clone L12-[IL-6R/IL-6] was isolated. This clone was found to be stable over many passages and semi-confluent cultures routinely secrete into the culture medium amounts of 2.5 µg/ml of the IL-6R/IL-6 chimera.

For purification of the IL-6R/IL-6 chimera, 3.25 liters of medium from clone L12 cultures in 2% bovine serum were concentrated to 200 ml. This was adsorbed on a 18 ml column of anti human sIL-6R Monoclonal Antibody 34.4 coupled to Affigel 10 beads and eluted as described (Novick et al., Hybridoma, 10, 137-146, 1991). A 25 mM citric acid eluate was immediately neutralized with 1 Hepes buffer pH 8.6. The proteins were concentrated on a 10 kDa cut-off Amicon membrane to a final concentration of 1 mg/ml. Upon SDS-PAGE, a single band of 85 kDa

corresponding to the IL-6R/IL-6 chimera was observed. Glycosylation was demonstrated by size reduction after treatment with glycosidase (Boehringer, Mannheim). The biological activity of the CHO produced IL-6R/IL-6 chimera was found stable for at least 5 months at 4°C. Routinely, storage is at -70°C.

5

#### Example 7 : Affinity of IL-6R/IL-6 chimera to gp130

CHO-produced IL-6R/IL-6 chimera and a mixture of human IL-6 and sIL-6R were compared for their binding to the soluble form of gp130 (sgp 130), which is the second chain of the receptor system for IL-6 (see background). A

10 microtiter 96-well plate (Nunc) was coated with anti-human gp130 monoclonal antibody and 50 ng/ml of sgp130 (both from R&D Systems, Minneapolis) was added. After washing in phosphate buffered saline, the IL-6R/IL-6 chimera was added in different wells at different concentrations ranging from 0.1 to 50 ng/ml. In separate wells, rhuIL-6 (Ares-Serono, Geneva) was added at 500 ng/ml together  
 15 with human sIL-6R $\delta$ Val at concentrations from 2 to 500 ng/ml. After incubation overnight at 4°C, a rabbit polyclonal anti-IL-6R (Oh et al., Cytokine, 8, 401-409, 1996) was added, followed by goat antirabbit Ig conjugated with horseradish peroxidase which was detected by colored reaction (Sigma, St. Louis). Figure 8 shows a Scatchard plot of the results. The affinity of the IL-6R/IL-6 chimera to  
 20 gp130 was found to be over 4 fold higher than that of the two parts of the molecule added separately ( $6.3 \times 10^{-11}$ M versus  $2.6 \times 10^{-10}$ M). This result is in line and explains the higher activity of the chimera as compared to the IL-6 + sIL-6R combination on melanoma and on hematopoietic cells (Figure 9 and Example 4).

25 Example 8 : The IL-6R/IL-6 chimera protects from hepatotoxicity

Carbon tetrachloride (CCl<sub>4</sub>) injection to mice produces a severe necrosis of the liver leading to death of the animals (Slater T.F. et al., Philos. Trans. R. Soc.

Biol. Sci. 311, 633-645, 1985). When mice which are genetically deficient in IL-6 (IL-6<sup>-/-</sup>) are given relatively low doses of CCl<sub>4</sub> (2-3 ml/kg body weight) by intraperitoneal injection, lethality rates at 24 hours are around 70% (Fig. 10). Injection of the CHO-produced IL-6R/IL-6 chimera one hour before CCl<sub>4</sub> and again 5 4 hours after CCl<sub>4</sub>, protects the animals and no deaths are seen at 24 hours. In contrast, free rhuIL-6 injected similarly has no effect (Fig. 10). The IL-6R/IL-6 chimera was effective at doses of 2-3 µg per injection, which in molar ratio are 10 times lower than the dose of IL-6, which was not effective. At higher doses of CCl<sub>4</sub> 10 (e.g. 3.5 ml/kg in Fig. 10), the chimera was also protective, the mortality being lower than with IL-6 or without cytokine. The difference in mortality between mice treated with chimera and untreated mice, both receiving the same CCl<sub>4</sub> challenge, was significant at p<0.01. Histological observation of liver sections stained with hematoxillin-eosin confirmed that CCl<sub>4</sub> produced liver tissue necrosis, and that 15 IL-6R/IL-6 chimera protects the hepatocytes from this chemical toxic effect (not shown).

An application of the IL-6R/IL-6 chimera may be for protection of liver tissue in patients with necrotic diseases due to chemicals (e.g. alcohol, paracetamol) or other causes (e.g. viral hepatitis).

20 **Example 9 : Construction and activity of IL-6/sIL-6RδVal chimera**

A chimeric molecule in which the IL-6 moiety is at the N-terminal whereas the sIL-6R moiety is at the C-terminal was constructed. Plasmid pBS-sIL-6RδVal was cut at Sau3a (bp 1086) and at the HindIII following the stop codon after Val-356 (see Example 1). A linker containing three restriction sites : SpeI, SmaI 25 and BamH1 was synthesized as follows :

SpeI	SmaI	BamH1
5' CT AGT GGG CCC GGG GTG GCG GG		
	A CCC GGG CCC CAC CGC CCC TAG 5'	(SEQ ID NO: 2)

5 This Sau3a site of sIL-6R was ligated to the BamH1 of the linker and cloned in the multiple cloning site of a Bluescript pBS SK plasmid. The IL-6 sequence was amplified by PCR from pKK $\beta$ 2-7 DNA using the primers (initiation codon underlined) :

10 SpeI  
Forward 5' GA CTA GTA GCT ATG AAC TCC TTC TC (SEQ ID NO:3)  
HaeIII  
Backward 5' AG GGC CAT TTG CCG AAG AGC C (SEQ ID NO:4)

15 The PCR product cut with SpeI and HaeIII was introduced between the SpeI and SmaI site of the above linker. Another linker BamH1-NcoI with an internal SmaI was synthesized as follows :

This was cloned between the BamH1 of the previous linker and the NcoI 1464 of the IL-6R sequence. A fragment of IL-6R from SmaI 867 to NcoI 1464 was then introduced between the SmaI of the second linker and the NcoI of IL-6R. The resulting chimeric DNA was sequenced and recloned in pCDNA3 for expression in human HEK 293 cells. The amino acid sequence of this IL-6-IL-6R chimera 3e is

shown in Figure 11 (linker underlined). Chimera 3e was purified by affinity chromatography on an anti-IL-6 monoclonal antibody (as in Novick et al., Hybridoma 8, 561-567, 1989). On SDS-PAGE, a 75 kDa band was observed.

The biological activity of the IL-6-IL-6R chimera 3e to inhibit the growth of  
5 the F10.9 melanoma cells is shown in Figure 12. It is clearly active as compared to  
the IL-6R/IL-6 chimera (preparation 1-3) in the same experiment, although more is  
required for 50% growth inhibition.

Two mutants of IL-6R/IL-6 were made in which amino acids His-280 and  
Asp-281 of the IL-6R moiety of IL-6R/IL-6 (Fig. 3) were changed to Ser and Val  
10 respectively by PCR mutagenesis (Mutant 39 or HD), or where Asn-230 was in  
addition changed to Asp (Mutant NHD). As can be seen from Figure 12, these two  
mutants had almost no activity as compared to the IL-6R/IL-6 and IL-6-IL-6R  
chimeras. Since in IL-6R, these amino acid interact with gp130, as shown by  
molecular modeling (Halimi et al., 1995), this demonstrates that the sIL-6R/IL-6  
15 chimera conserves this essential interaction site.

The IL-6-IL-6R chimera 3e is missing the immunoglobulin-like domain of  
IL-6R which is present in IL-6R/IL-6. However, just removing this Ig-domain from  
IL-6R/IL-6 did not reduce its biological activity on F10.9 cells. The binding of  
IL-6-IL-6R chimera 3e to gp130 was about 30% of that of another IL-6R/IL-6  
20 chimera (not shown). This lower binding is in line with the lower activity on the  
melanoma cell growth.

These results demonstrate that the blocking of IL-6 carboxyterminus by  
fusion through a linker to sIL-6R, conserves a good biological activity in such novel  
chimeras.

References

- Chen L, Mory Y, Zilberstein A and Revel M. Growth inhibition of human breast carcinoma and leukemia/lymphoma cell lines by recombinant interferon-beta 2/IL-6. Proc. Natl. Acad. Sci. USA, 85: 8037-8041, 1988.
- Chernajovsky Y, Mory Y, Chen L, Marks Z, Novick D, Rubinstein M and Revel M. Efficient constitutive production of human fibroblast interferon by hamster cells transformed with the IFN- $\beta$ 1 gene fused to an SV40 early promoter. DNA, 3: 297-308, 1984.
- Fischer M, Goldschmitt J, Peschel C, Brakenhoff JPG, Kallen K-J, Wollmer A, Grotzinger J and Rose-John S. A bioactive designer cytokine for human hematopoietic progenitor cell expansion. Nature Biotechnology 15: 142-145, 1997.
- Ganapathi MK, Weizer AK, Borsellino S, Bukowski RM, Ganapathi S, Rice T, Casey G and Kawamura K. Resistance to Interleukin-6 in human Non-small cell lung carcinoma cell lines: Role of receptor components. Cell Growth and Differentiation, 7: 923-929, 1996.
- Halimi H, Eisenstein M, Oh J, Revel M and Chebath J. Epitope peptides from interleukin-6 receptor which inhibit the growth of human myeloma cells. Eur. Cytokine Netw., 6: 135-143, 1995.
- Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashimura S, Nakajima K, Koyama K, Iwamatsu K, Tsunasawa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T and Kishimoto T. Complementary DNA for a novel interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulins. Nature, 234: 73-76, 1986.
- Hirano T, Matsuda T and Nakajima K. Signal transduction through gp130 that is shared among the receptors for the interleukin 6 related cytokine subfamily. Stem cells, 12:262-277, 1994.

- Holloway CJ. Applications of recombinant DNA technology in the production of glycosylated recombinant human granulocyte colony stimulating factor. Eur. J. Cancer, 30: S2-6, 1994.
- Kahn MA and De Vellis J. Regulation of an oligodendrocyte progenitor cell line by 5 the interleukin-6 family of cytokines. Glia, 12: 87-98, 1994.
- Katz A, Shulman LM, Porgador A, Revel M, Feldman M and Eisenbach L. Abrogation of B16 melanoma metastases by long-term low-dose Interleukin-6 therapy. J. Immunother. 13: 98-109, 1993.
- Mackiewicz A, Wiznerowicz M, Roeb E, Nowak J, Pawlowski T, Baumann H, 10 Heinrich P and Rose-John S. Interleukin-6-type cytokines and their receptors for gene therapy of melanoma. Ann. New York Acad. Sci., 762: 361-374, 1995.
- McKenna HJ, de Vries P, Brasel K, Lyman SD and Williams DE. Effect of flt3 ligand on the ex vivo expansion of human CD34+ hematopoietic progenitor cells. Blood 86: 3413-3420, 1995.
- Murakami M, Hibi M, Nakagawa N, Nagakawa T, Yasukawa K, Yamanishi K, Taga T and Kishimoto T. IL-6 induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science, 260: 1808-1810, 1993.
- Novick D, Englemann H, Wallach D, Leitner O, Revel M and Rubinstein M. Purification of soluble cytokine receptors from normal urine by ligand-affinity and 20 immunoaffinity chromatography. J. Chromatogr., 510: 331-337, 1990.
- Novick D, Engelmann H, Revel M, Leitner O and Rubinstein M. Monoclonal antibodies to the soluble IL-6 receptor: affinity purification, ELISA and inhibition of ligand binding. Hybridoma, 10: 137-146, 1991.
- Novick D, Shulman LM, Chen L and Revel M. Enhancement of interleukin-6 25 cytostatic effect on human breast carcinoma cells by soluble IL-6 receptor from urine and reversion by monoclonal antibodies. Cytokine, 4: 6-11, 1992.

- Oh J-W, Revel M and Chebath J. A soluble interleukin-6 receptor isolated from conditioned medium of human breast cancer cells is encoded by a differentially spliced mRNA. *Cytokine*, 8: 401-409, 1996.
- Oh J-W. Expression of recombinant soluble human interleukin-6 receptors and analysis of their functions. Ph.D. Thesis Weizmann Institute of Science (Revel M, supervisor), 1997.
- Paonessa G, Graziani R, DeSerio A, Savino R, Ciapponi L, Lahmm A, Salvati AL, Toniatti C and Ciliberto G. Two distinct and independent sites on IL-6 trigger gp130 dimer formation and signalling. *EMBO J.*, 14: 1942-1951, 1995.
- 10 Revel M. Host defense against infections and inflammations: Role of the multifunctional IL-6/IFN- $\beta$ 2 cytokine. *Experientia* 45: 549-557, 1989.
- Sambrook J, Fritsch EF and Maniatis T. Molecular cloning: A laboratory manual. Cold Spring Harbor Press, 1989.
- Sui X, Tsuji K, Tanaka R, Tajima S, Muraoka K, Ebihara Y, Ikebuchi K, Yasukawa 15 K, Taga T, Kishimoto T and Nakahata T. Gp130 and c-kit signalings synergize for ex vivo expansion of human primitive hemopoietic progenitor cells. *Proc. Natl. Acad. Sci. USA* 92: 2859-2863, 1995.
- Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T and Kishimoto T. Interleukin-6 triggers the association of its receptor with a possible 20 signal transducer gp130. *Cell*, 58: 573-581, 1989.
- Vormoor J, Lapidot T, Pflumio F, Risdon G, Patterson B, Broxmeyer HE and Dick JE. SCID mice as an in vivo model of human cord blood hematopoiesis. *Blood cells* 20: 316-320, 1994.
- Ward LD, Howlett GJ, Discolo G, Yasukawa K, Hammacher A, Moritz RL and 25 Simpson RJ. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor and gp130. *J. Biol. Chem.*, 269: 23286-23289, 1994.

- Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T and Kishimoto T. Cloning and expression of the human Interleukin-6 (BSF-2/ Interferon beta-2) receptor. *Science*, 241: 825-828, 1988.
- 5 Zilberstein A, Ruggieri R, Korn HJ and Revel M. Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth-stimulatory cytokines. *EMBO J.*, 5: 2529-2537, 1986.

0003-941X(199003)10:3;1-2

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Yeda Research and Development Co. Ltd.
  - (B) STREET: Weizmann Institute of Science, P.O.B. 95
  - (C) CITY: Rehovot
  - (E) COUNTRY: Israel
  - (F) POSTAL CODE (ZIP): 76100
  - (G) TELEPHONE: 972-8-9344093
  - (H) TELEFAX: 972-8-9470739
- 
- (A) NAME: REVEL, Michel
  - (B) STREET: Beit Brazil 5, Weizmann Institute of Science
  - (C) CITY: Rehovot
  - (E) COUNTRY: Israel
  - (F) POSTAL CODE (ZIP): 76100
- 
- (A) NAME: CHEBATH, Judith
  - (B) STREET: Rehov Miller 13
  - (C) CITY: Rehovot
  - (E) COUNTRY: Israel
  - (F) POSTAL CODE (ZIP): 76284
- 
- (A) NAME: LAPIDOT, Tsvee
  - (B) STREET: Rehov Boxer 6
  - (C) CITY: Ness-Ziona
  - (E) COUNTRY: Israel
  - (F) POSTAL CODE (ZIP): 74046
- 
- (A) NAME: KOLLET, Orit
  - (B) STREET: Rehov Ramat Chen 14
  - (C) CITY: Ramat Gan
  - (E) COUNTRY: Israel
  - (F) POSTAL CODE (ZIP): 52232

(ii) TITLE OF INVENTION: Chimeric Interleukin-6 Soluble Receptor/Ligand Protein, Analogs thereof and Uses thereof

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: IL 121284
- (B) FILING DATE: 10-JUL-1997

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: IL 122818
- (B) FILING DATE: 30-DEC-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Glu Phe Gly Ala Gly Leu Val Leu Gly Gly Gln Phe Met  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTAGTGGGCC CGGGGTGGCG GGACCCGGGC CCCACCGCCC CTAG

44

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GACTAGTAGC TATGAACTCC TTCTC

25

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGGCCATTT GCCGAAGAGC C

21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATCCGGGCG GCGGGGGAGG GGGGCCCGGG CGCCCCGCAGC CCCCTCCTCC CGGGCCCGGT

60

AC

62

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Gly Gly Gly Asp Pro Gly Gly Gly Gly Gly Pro Gly  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 543 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Leu Ala Val Gly Cys Ala Leu Leu Ala Ala Leu Leu Ala Ala Pro  
1 5 10 15

Gly Ala Ala Leu Ala Pro Arg Arg Cys Pro Ala Gln Glu Val Ala Arg

20

25

30

Gly Val Leu Thr Ser Leu Pro Gly Asp Ser Val Thr Leu Thr Cys Pro  
 35 40 45

Gly Val Glu Pro Glu Asp Asn Ala Thr Val His Trp Val Leu Arg Lys  
 50 55 60

Pro Ala Ala Gly Ser His Pro Ser Arg Trp Ala Gly Met Gly Arg Arg  
 65 70 75 80

Leu Leu Leu Arg Ser Val Gln Leu His Asp Ser Gly Asn Tyr Ser Cys  
 85 90 95

Tyr Arg Ala Gly Arg Pro Ala Gly Thr Val His Leu Leu Val Asp Val  
 100 105 110

Pro Pro Glu Glu Pro Gln Leu Ser Cys Phe Arg Lys Ser Pro Leu Ser  
 115 120 125

Asn Val Val Cys Glu Trp Gly Pro Arg Ser Thr Pro Ser Leu Thr Thr  
 130 135 140

Lys Ala Val Leu Leu Val Arg Lys Phe Gln Asn Ser Pro Ala Glu Asp  
 145 150 155 160

Phe Gln Glu Pro Cys Gln Tyr Ser Gln Glu Ser Gln Lys Phe Ser Cys  
 165 170 175

Gln Leu Ala Val Pro Glu Gly Asp Ser Ser Phe Tyr Ile Val Ser Met  
 180 185 190

Cys Val Ala Ser Ser Val Gly Ser Lys Phe Ser Lys Thr Gln Thr Phe  
 195 200 205

Gln Gly Cys Gly Ile Leu Gln Pro Asp Pro Pro Ala Asn Ile Thr Val  
 210 215 220

Thr Ala Val Ala Arg Asn Pro Arg Trp Leu Ser Val Thr Trp Gln Asp  
 225 230 235 240

Pro His Ser Trp Asn Ser Ser Phe Tyr Arg Leu Arg Phe Glu Leu Arg  
 245 250 255

Tyr Arg Ala Glu Arg Ser Lys Thr Phe Thr Thr Trp Met Val Lys Asp  
 260 265 270

Leu Gln His His Cys Val Ile His Asp Ala Trp Ser Gly Leu Arg His  
 275 280 285

Val Val Gln Leu Arg Ala Gln Glu Glu Phe Gly Gln Gly Glu Trp Ser  
 290 295 300

Glu Trp Ser Pro Glu Ala Met Gly Thr Pro Trp Thr Glu Ser Arg Ser  
 305 310 315 320

Pro Pro Ala Glu Asn Glu Val Ser Thr Pro Met Gln Ala Leu Thr Thr  
 325 330 335

Asn Lys Asp Asp Asp Asn Ile Leu Phe Arg Asp Ser Ala Asn Ala Thr  
 340 345 350

DRAFT - 04/10/2008

Ser Leu Pro Val Glu Phe Met Pro Val Pro Pro Gly Glu Asp Ser Lys  
 355 360 365  
 Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg Ile  
 370 375 380  
 Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys  
 385 390 395 400  
 Glu Thr Cys Asn Lys Ser Asn Met Cys Glu Ser Ser Lys Glu Ala Leu  
 405 410 415  
 Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu Lys Asp Gly Cys  
 420 425 430  
 Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val Lys Ile Ile Thr  
 435 440 445  
 Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln Asn Arg Phe  
 450 455 460  
 Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser Thr Lys Val  
 465 470 475 480  
 Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr  
 485 490 495  
 Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr Lys Leu Gln Ala  
 500 505 510  
 Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile Leu Arg Ser  
 515 520 525  
 Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg Gln Met  
 530 535 540

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 471 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Asn Ser Phe Ser Thr Ser Ala Phe Gly Pro Val Ala Phe Ser Leu  
 1 5 10 15  
 Gly Leu Leu Leu Val Leu Pro Ala Ala Phe Pro Ala Pro Val Pro Pro  
 20 25 30  
 Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr  
 35 40 45

Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile  
 50 55 60

Ser Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser Asn Met Cys Glu Ser  
 65 70 75 80

Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala  
 85 90 95

Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu  
 100 105 110

Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr  
 115 120 125

Leu Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln  
 130 135 140

Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn  
 145 150 155 160

Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu  
 165 170 175

Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His  
 180 185 190

Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala  
 195 200 205

Leu Arg Gln Met Gly Gly Gly Asp Pro Gly Gly Gly Gly Gly Gly  
 210 215 220

Pro Gly Val Pro Pro Glu Glu Pro Gln Leu Ser Cys Phe Arg Lys Ser  
 225 230 235 240

Pro Leu Ser Asn Val Val Cys Glu Trp Gly Pro Arg Ser Thr Pro Ser  
 245 250 255

Leu Thr Thr Lys Ala Val Leu Leu Val Arg Lys Phe Gln Asn Ser Pro  
 260 265 270

Ala Glu Asp Phe Gln Glu Pro Cys Gln Tyr Ser Gln Glu Ser Gln Lys  
 275 280 285

Phe Ser Cys Gln Leu Ala Val Pro Glu Gly Asp Ser Ser Phe Tyr Ile  
 290 295 300

Val Ser Met Cys Val Ala Ser Ser Val Gly Ser Lys Phe Ser Lys Thr  
 305 310 315 320

Gln Thr Phe Gln Gly Cys Gly Ile Leu Gln Pro Asp Pro Pro Ala Asn  
 325 330 335

Ile Thr Val Thr Ala Val Ala Arg Asn Pro Arg Trp Leu Ser Val Thr  
 340 345 350

Trp Gln Asp Pro His Ser Trp Asn Ser Ser Phe Tyr Arg Leu Arg Phe  
 355 360 365

Glu Leu Arg Tyr Arg Ala Glu Arg Ser Lys Thr Phe Thr Thr Trp Met

370

375

380

Val Lys Asp Leu Gln His His Cys Val Ile His Asp Ala Trp Ser Gly  
385 390 395 400

Leu Arg His Val Val Gln Leu Arg Ala Gln Glu Glu Phe Gly Gln Gly  
405 410 415

Glu Trp Ser Glu Trp Ser Pro Glu Ala Met Gly Thr Pro Trp Thr Glu  
420 425 430

Ser Arg Ser Pro Pro Ala Glu Asn Glu Val Ser Thr Pro Met Gln Ala  
435 440 445

Leu Thr Thr Asn Lys Asp Asp Asn Ile Leu Phe Arg Asp Ser Ala  
450 455 460

Asn Ala Thr Ser Leu Pro Val  
465 470

**CLAIMS**

1. A chimeric glycosylated soluble interleukin-6 receptor (sIL-6R)-interleukin-6 (IL-6) protein (sIL-6R/IL-6) and biologically active analogs thereof, comprising a fusion protein product between essentially all of the naturally occurring form of sIL-6R and essentially all of the naturally occurring form of IL-6, said sIL-6R/IL-6 and analogs thereof being glycosylated in a similar fashion to the glycosylation of naturally occurring sIL-6R and IL-6.

2. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to claim 1, wherein said sIL-6R is fused to IL-6 via a peptide linker molecule.

3. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to claim 2, wherein said linker is a very short, non-immunogenic linker of about 3 amino acid residues.

4. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to claim 3, wherein said linker is a tripeptide of the sequence E-F-M (Glu-Phe-Met).

5. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to claim 2, wherein said linker is a peptide of 13 amino acid residues of sequence E-F-G-A-G-L-V-L-G-G-Q-F-M (Glu-Phe-Gly-Ala-Gly-Leu-Val-Leu-Gly-Gly-Gln-Phe-Met).

6. A chimeric sIL-6R/IL-6 protein according to any one of claims 1-4, being the herein designated sIL-6R $\delta$ Val/IL-6 having a tripeptide linker of sequence

E-F-M between the C-terminal Val-356 of sIL-6R and the N-terminal Pro-29 of IL-6R, said chimeric protein having the sequence set forth in Fig. 3.

7. A chimeric sIL-6R/IL-6 protein according to any one of claims 1, 2, and 5,  
5 being the herein designated sIL-6R $\delta$ Val/L/IL-6 having a 13 amino acid peptide  
linker of sequence E-F-G-A-G-L-V-L-G-G-Q-F-M between the C-terminal Val-356  
of sIL-6R and the N-terminal Pro-29 of IL-6R, said chimeric protein having the  
sequence set forth in Fig. 3 wherein the tripeptide of sequence E-F-M between  
positions 357-359 of Fig. 3 is replaced by said 13 amino acid peptide sequence.

10

8. A chimeric sIL-6R/IL-6 protein according to claim 1 being the herein  
designated IL-6/sIL-6R having the entire sequence of IL-6 preceding the sIL-6R  
sequence with a 14 amino acid peptide linker of sequence  
G-G-G-G-D-P-G-G-G-G-G-P-G (SEQ ID NO: 6) between the C-terminal  
15 MET-212 of IL-6 and the VAL-112 of sIL-6R, said chimeric protein having the  
sequence set forth in Fig. 11.

9. A chimeric sIL-6R/IL-6 protein according to any one of claims 1-8, wherein  
said protein is produced in mammalian cells in a fully processed form.

20

10. A chimeric sIL-6R/IL-6 protein according to claim 9, wherein said protein is  
produced in human cells.

25

11. A chimeric sIL-6R/IL-6 protein according to claim 9, wherein said protein is  
produced in CHO cells.

12. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to any one of claims 1-11, wherein said chimeric protein and analogs are characterized by being capable of inhibiting the growth of highly malignant cancer cells.

5

13. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to claim 12, wherein said chimeric protein and analogs are characterized by being capable of inhibiting the growth of highly malignant melanoma cells.

DOCKET NUMBER: 09-10000

10 14. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to any one of claims 1-11, wherein said chimeric protein and analogs are characterized by being capable of eliciting the *in vivo* engraftment of human hematopoietic cells in bone marrow transplantations.

15 15. A chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to any one of claims 1-11, wherein said chimeric protein and analogs are characterized by being capable of protecting liver from hepatotoxic agents.

16. A DNA sequence encoding a chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to any one of claims 1-11.

20  
25 17. A DNA vector comprising a DNA sequence encoding a chimeric sIL-6R/IL-6 protein and biologically active analogs thereof according to any one of claims 1-11, said vector being suitable for expression of said chimeric protein in mammalian cells.

18. A DNA vector according to claim 17, wherein said vector is suitable for expression of said chimeric protein in human cells.

19. A DNA vector according to claim 17, wherein said vector is suitable for  
5 expression of said chimeric protein in CHO cells.

20. A DNA vector according to claim 17-19, wherein when said vector is expressed in mammalian or human cells, the expressed chimeric protein has a sequence that permits full processing of the chimeric protein by the mammalian or  
10 human cells and secretion of the fully processed chimeric protein from the cells into the culture medium in which said cells are grown.

21. A DNA vector according to any one of claims 17-20, wherein said vector is the herein designated plasmid pcDNAsIL-6R/IL-6 comprising a pcDNA3 vector  
15 containing the DNA sequence encoding the chimeric sIL-6R/IL-6 protein under the control of a cytomegalovirus (CMV) promoter.

22. A DNA vector according to any one of claims 17-20, wherein said vector is the herein designated plasmid pcDNA sIL-6R/L/IL-6 comprising a pcDNA3 vector  
20 containing the DNA sequence encoding the chimeric sIL-6R/IL-6 protein under the control of a cytomegalovirus (CMV) promoter, and wherein in said DNA sequence encoding said chimeric sIL-6R/IL-6 protein there is inserted a linker sequence encoding a peptide linker at the EcoRI site placed between the sequence encoding the sIL-6R part and the sequence encoding the IL-6 part of the protein.

25  
23. Transformed mammalian cells containing a DNA vector according to any one of claims 17-22 which are capable of expressing the sIL-6R/IL-6 chimeric

DOCUMENT NUMBER: 00000000000000000000000000000000

protein sequence carried by said vector and of fully processing the expressed protein and secreting it into the culture medium in which said cells are grown.

24. Transformed cells according to claim 23 wherein in said cells are the herein  
5 described human embryonal kidney cells 293 (HEK293) transfected by the pcDNA  
sIL-6R/IL-6 vector, said cells being capable of expressing the sIL-6R/IL-6 chimeric  
protein, fully processing said protein and secreting said protein into the culture  
medium in which said cells are grown in the form of an about 85 kDa glycoprotein.

10 25. A method for producing a chimeric protein or biologically active analogs  
thereof according to any one of claims 1-14, comprising growing transformed cells  
according to claim 23 or 24 under conditions suitable for expression, processing  
and secretion of said protein or analogs into the culture medium in which said cells  
are grown; and purifying said protein or analogs from said culture medium.

15 26. A method according to claim 25, wherein the purification is carried out by  
immunoaffinity chromatography using monoclonal antibodies specific for sIL-6R.

27. The use of a chimeric sIL-6R/IL-6 protein or analogs according to any one of  
20 claims 1-11, salts of any one thereof, and mixtures thereof, as an inhibitor of cancer  
cells.

28. The use of a chimeric protein or analog according to claim 27, as an inhibitor  
of highly malignant melanoma cells.

25 29. The use of a chimeric sIL-6R/IL-6 protein or analogs according to any one of  
claims 1-11, salts of any one thereof, and mixtures thereof, as an active ingredient

for eliciting engraftment of human hematopoietic cells in bone marrow transplantation.

30. The use of a chimeric sIL-6R/IL-6 protein or analogs according to any one of  
5 claims 1-11, salts of any one thereof, and mixtures thereof, as an active ingredient  
for protecting liver against hepatotoxic agents.

31. The use of a chimeric sIL-6R/IL-6 protein or analogs according to any one of  
10 claims 1-11, salts of any one thereof, and mixtures thereof, as an active ingredient  
for increasing hematopoiesis, for treating liver or neurological conditions, or for  
other applications in which IL-6 or sIL-6R are used.

32. A chimeric sIL-6R/IL-6 protein or analogs according to any one of claims  
15 1-11, salts of any one thereof and mixtures thereof, for use in the preparation of a  
medicament for treating mammalian cancers by way of inhibition of mammalian  
cancer cells, or in the preparation of a medicament for enhancement of bone  
marrow transplantation by way of eliciting engraftment of human hematopoietic  
cells in bone marrow transplantation, or in the preparation of a medicament for  
increasing hematopoeisis, or in the preparation of a medicament for treating liver or  
20 neurological disorders, or in the preparation of a medicament for other applications  
in which IL-6 or sIL-6R are used.

33. A pharmaceutical composition comprising as active ingredient a chimeric  
25 sIL-6R/IL-6 protein or analog thereof according to any one of claims 1-11, and a  
pharmaceutically acceptable carrier, diluent or excipient.

34. A pharmaceutical composition according to claim 33 for the treatment of cancers.

35. A pharmaceutical composition according to claim 33 for the enhancement of  
5 bone marrow transplantation.

36. A pharmaceutical composition according to claim 33 for the treatment of liver or neurological disorders, or for increasing hematopoeisis or for other applications in which IL-6 or sIL-6R are used.

10  
37. A method for treating cancers in mammals, or for enhancing bone marrow transplantations, or for treating liver or neurological disorders, or for increasing hematopoiesis, or for other applications in which IL-6 or sIL-6R are used, comprising administering to a patient a pharmaceutical composition according to  
15 any one of claims 33-36 in a suitable dosage form and by a suitable route of administration.

20

25

DOCUMENT ID: 04416200

**ABSTRACT**

Chimeric proteins constructed from the fusion of the naturally occurring  
5 form of the soluble IL-6 receptor and IL-6 which are useful for treatment of cancer  
and liver disorders, enhancement of bone marrow transplantation, and treatment of  
other IL-6 related conditions are provided.

09/462416

1/15

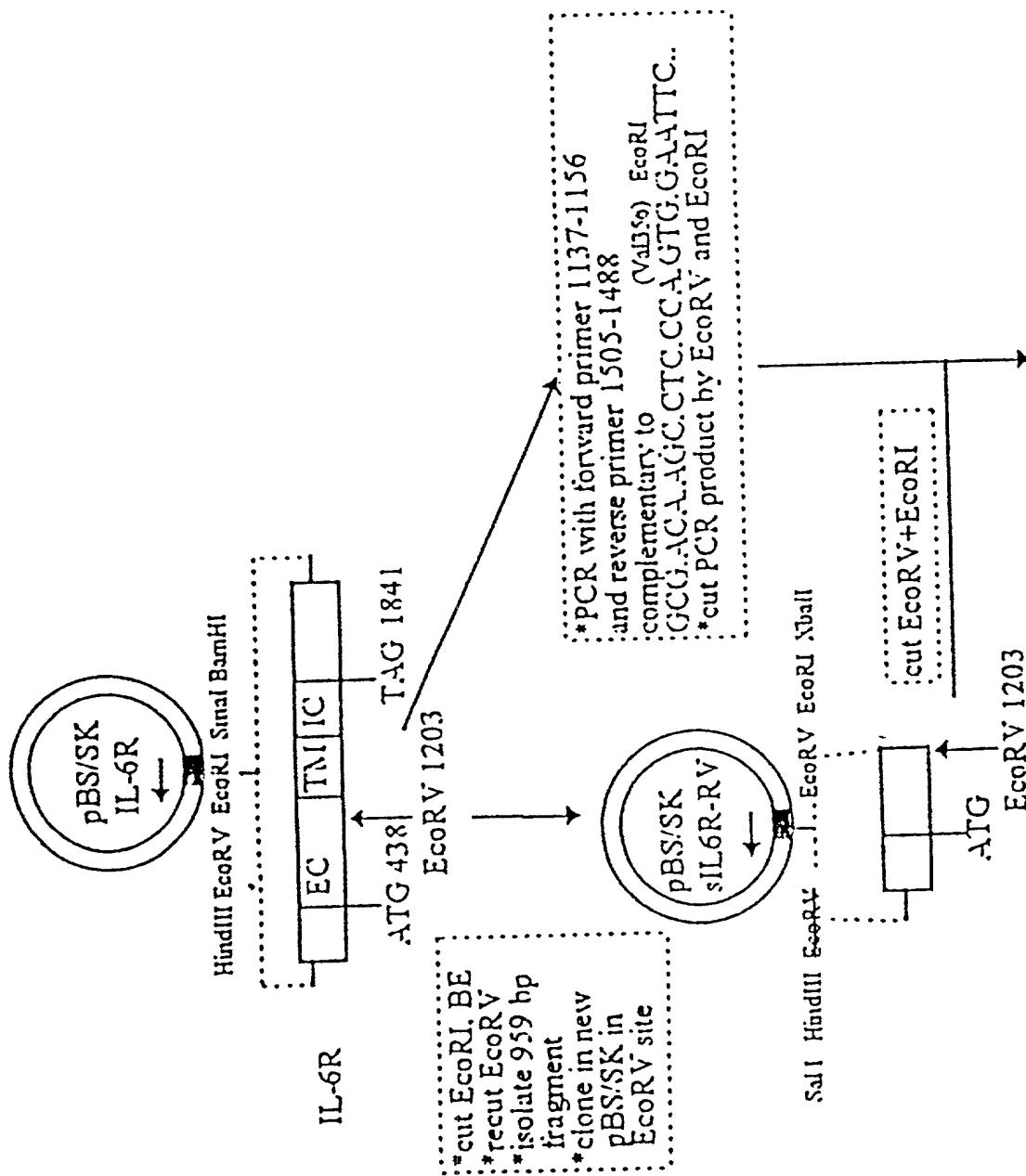
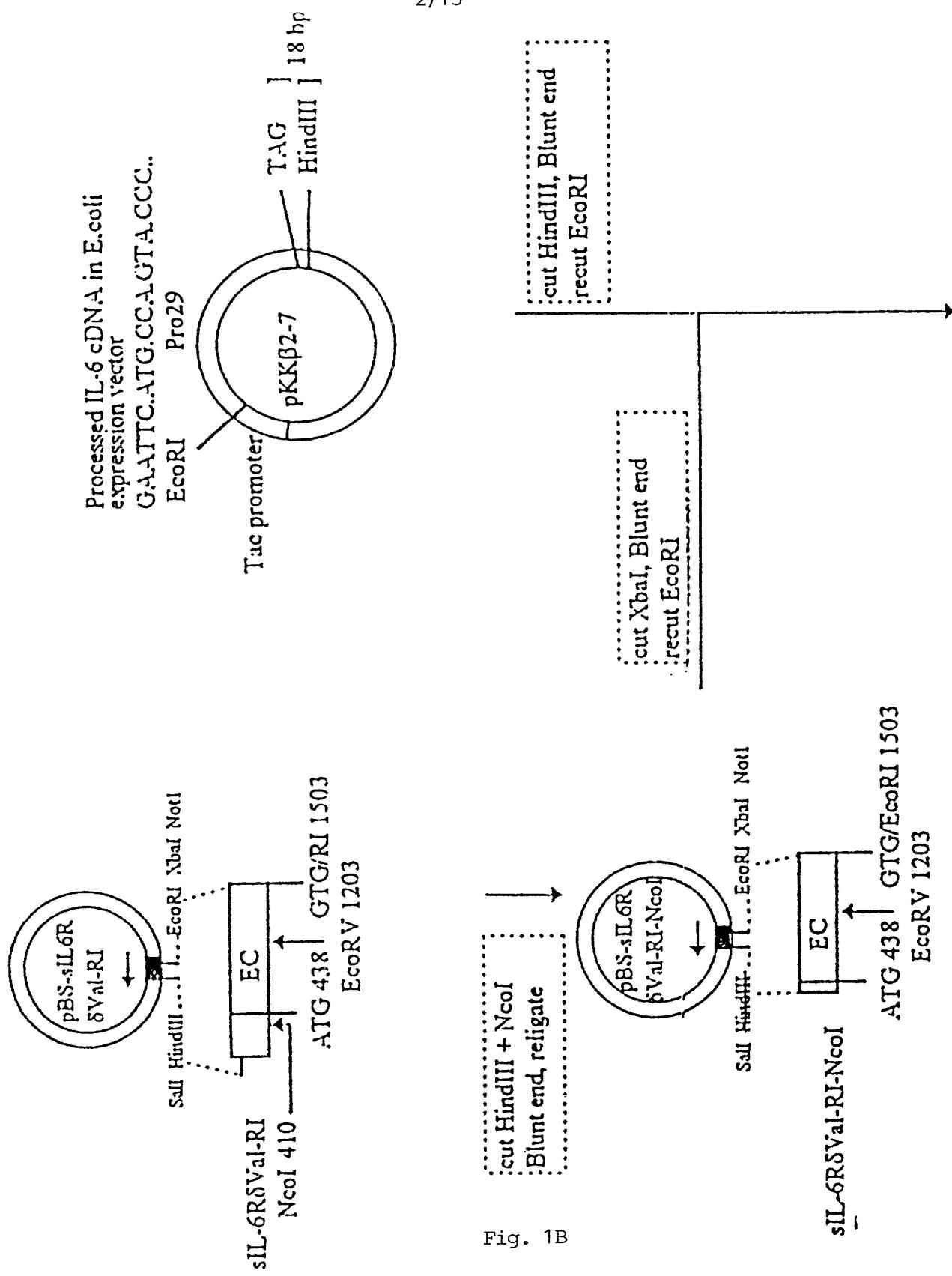


Fig. 1A

2/15



3/15

© 1999 Elsevier Science Ltd. All rights reserved.

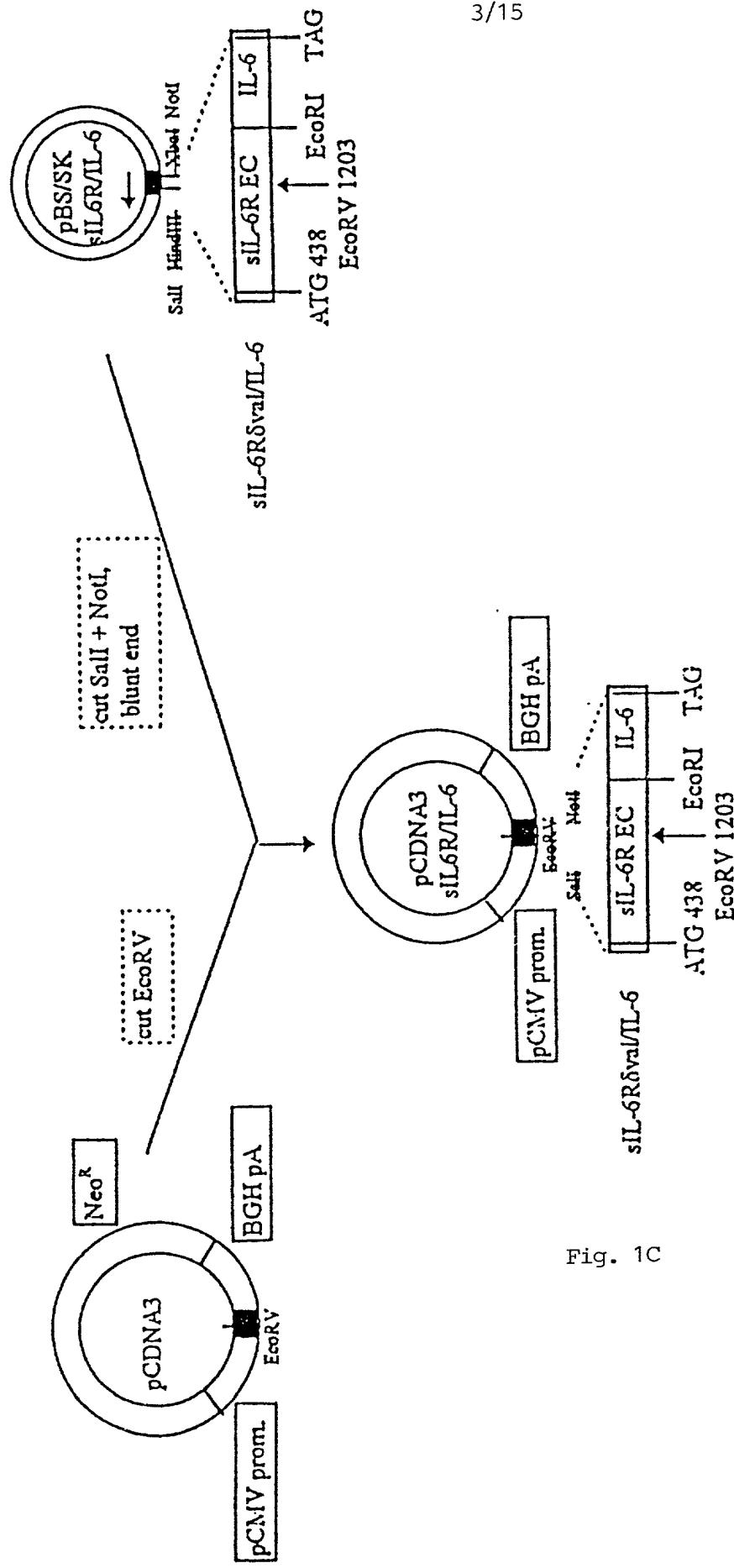


Fig. 1C

09/462416

4/15

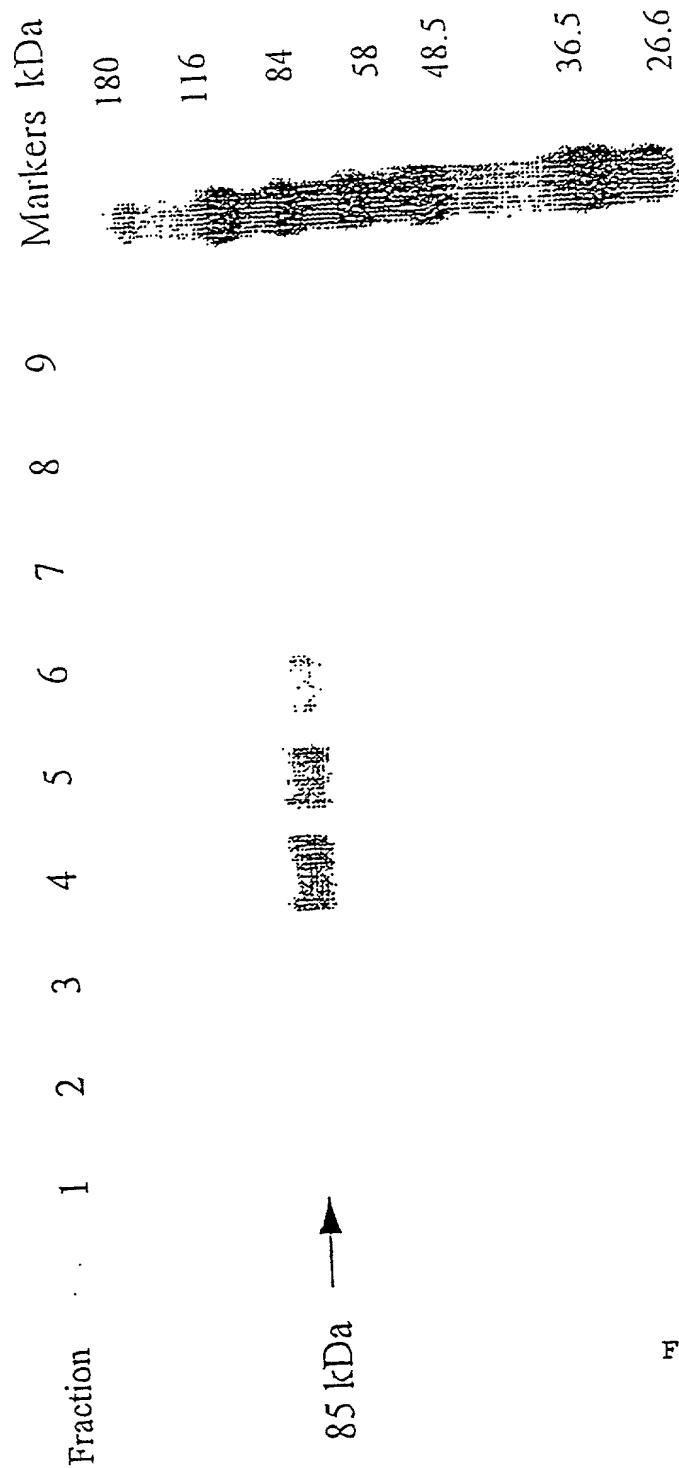
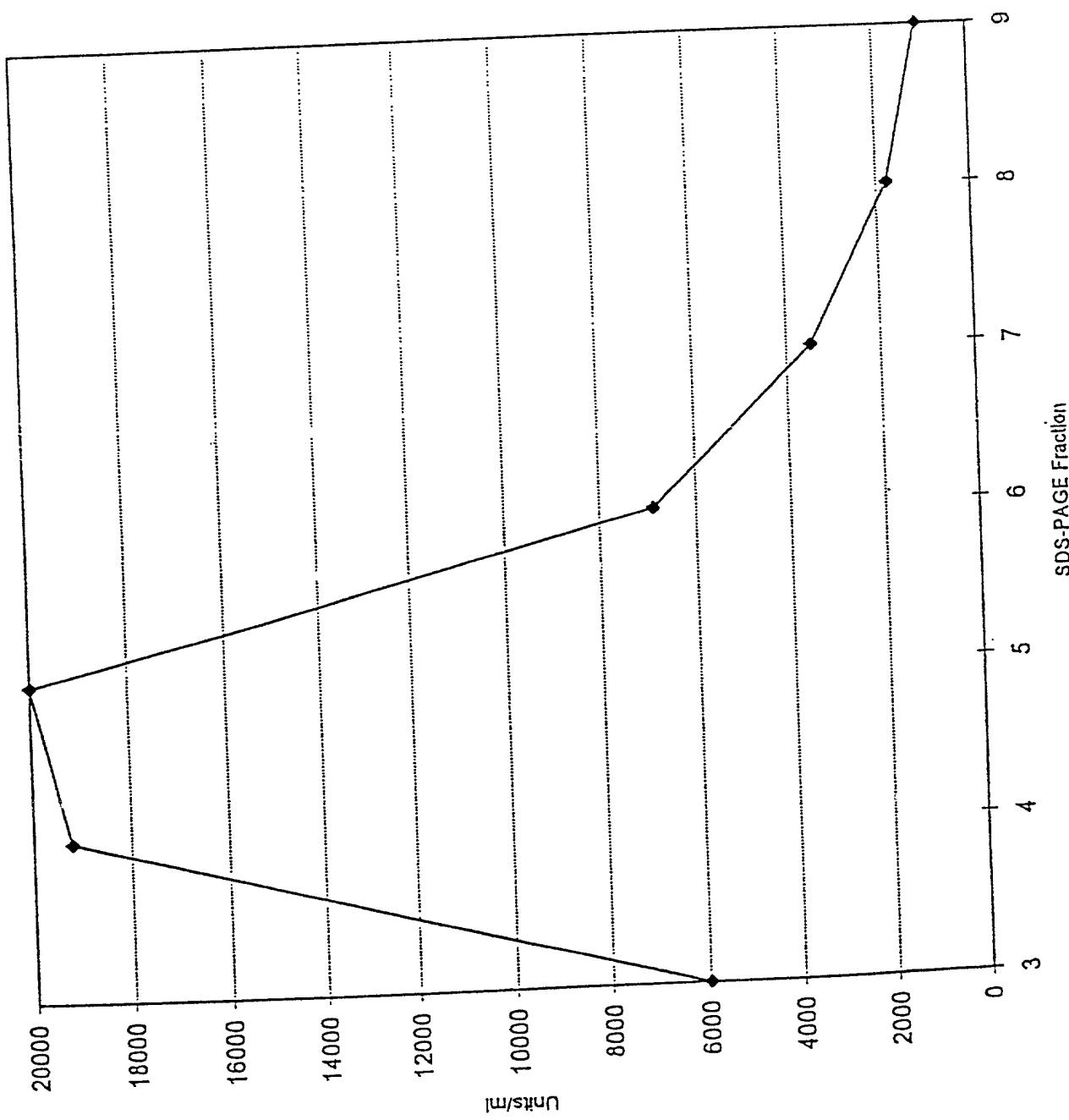


Fig. 2A

09/462416

5/15

Fig. 2B



6/15

. 10 . 20 . 30 . 40 . 50 . 60  
MLAVGCALLAALLAAPGAALAPRRCPAQEVARGVLTS  
 signal peptide Ig-like domain  
 . 70 . 80 . 90 . 100 . 110 . 120  
VLRKPAAGSHPSRWAGMGRLLLRSVQLHDSGNYSCYRAGR  
PAGTVHLLVDVPPPEEPOLS

. 130 . 140 . 150 . 160 . 170 . 180  
CFRKSPLSNVVCEWGPRTSPSLTTKAVLLVRKFONSPAEDFOEPCOYSQESOKFSCOLAV  
 cytokine receptor N-domain  
 . 190 . 200 . 210 . 220 . 230 . 240  
PEGDSSFYIVSMCVASSVGSKFSKTOTFOCGILOPDPPANITVTAVARNPRWLSVTWQD

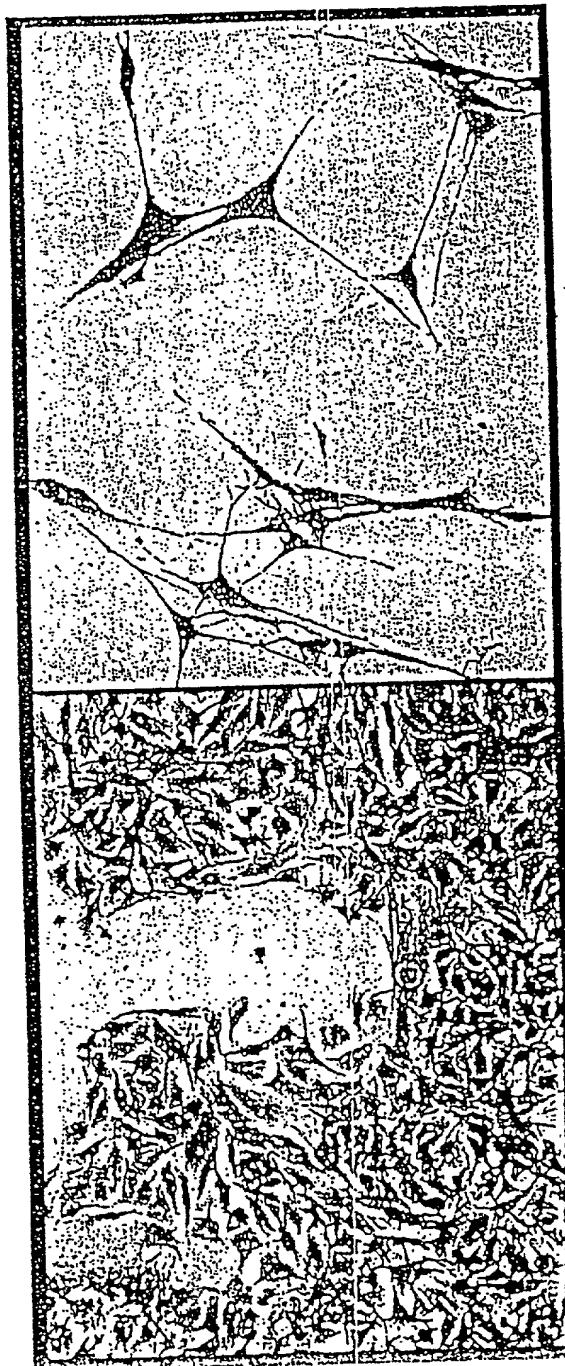
. 250 . 260 . 270 . 280 . 290 . 300  
PHSWNSSFYRLRFELRYRAERSKTFTTWMVKDLQHHCVIHDAWSGLRHVVQLRAQEEFGQ  
 cytokine receptor C-domain  
 . 310 . 320 . 330 . 340 . 350 . 360  
GEWSEWSPEAMGTPWTESRSPPAENEVSTPMQALTNKDDDNILFRDSANATSLPVEFMP  
 receptor pre-membrane region  
 . 370 . 380 . 390 . 400 . 410 . 420  
VPPGEDSKDVAAAPHROPLTSSERIDKOIRYILDGISALRKETCNKSNMCESSKEALAENN  
 IL-6  
 . 430 . 440 . 450 . 460 . 470 . 480  
LNLPKMAEKDGCFOSGFNEETCLVKIITGLLEFEVYLEYLONRFESSEEQARAVOMSTKV

. 490 . 500 . 510 . 520 . 530 . 540  
LIQFLOKKAKNLDAITTPDPTTNASLLTKLOAONOWLODMTHLILRSFKEFLOSSLRALROM

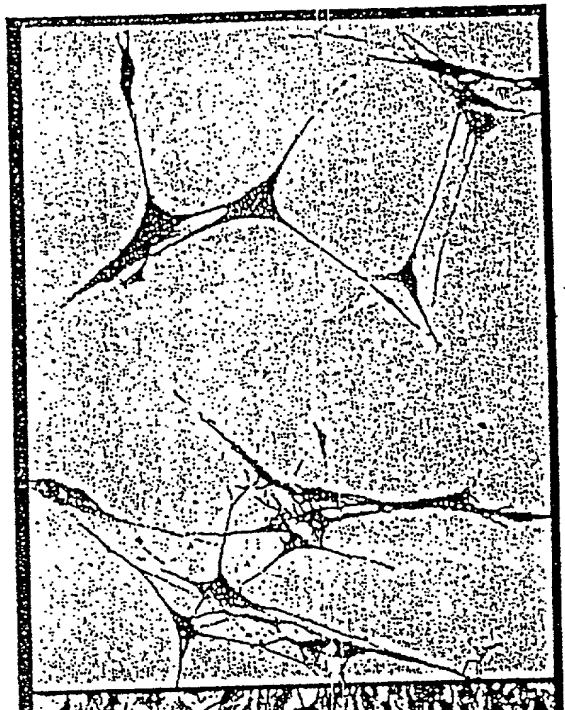
Fig. 3

09/462416

7/15



A



B

Fig. 4

09/462416

8/15

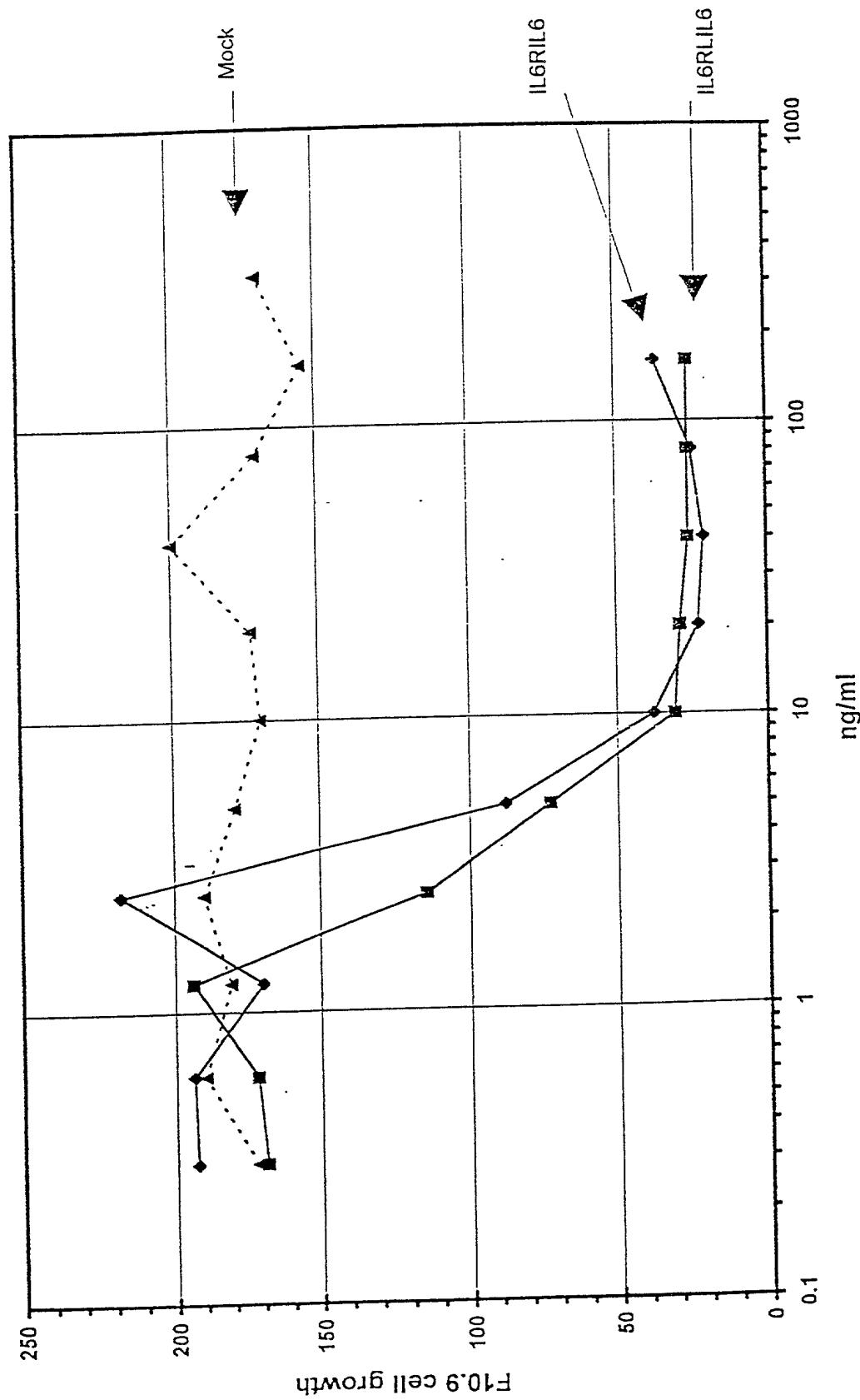


Fig. 5

09/462416

9/15

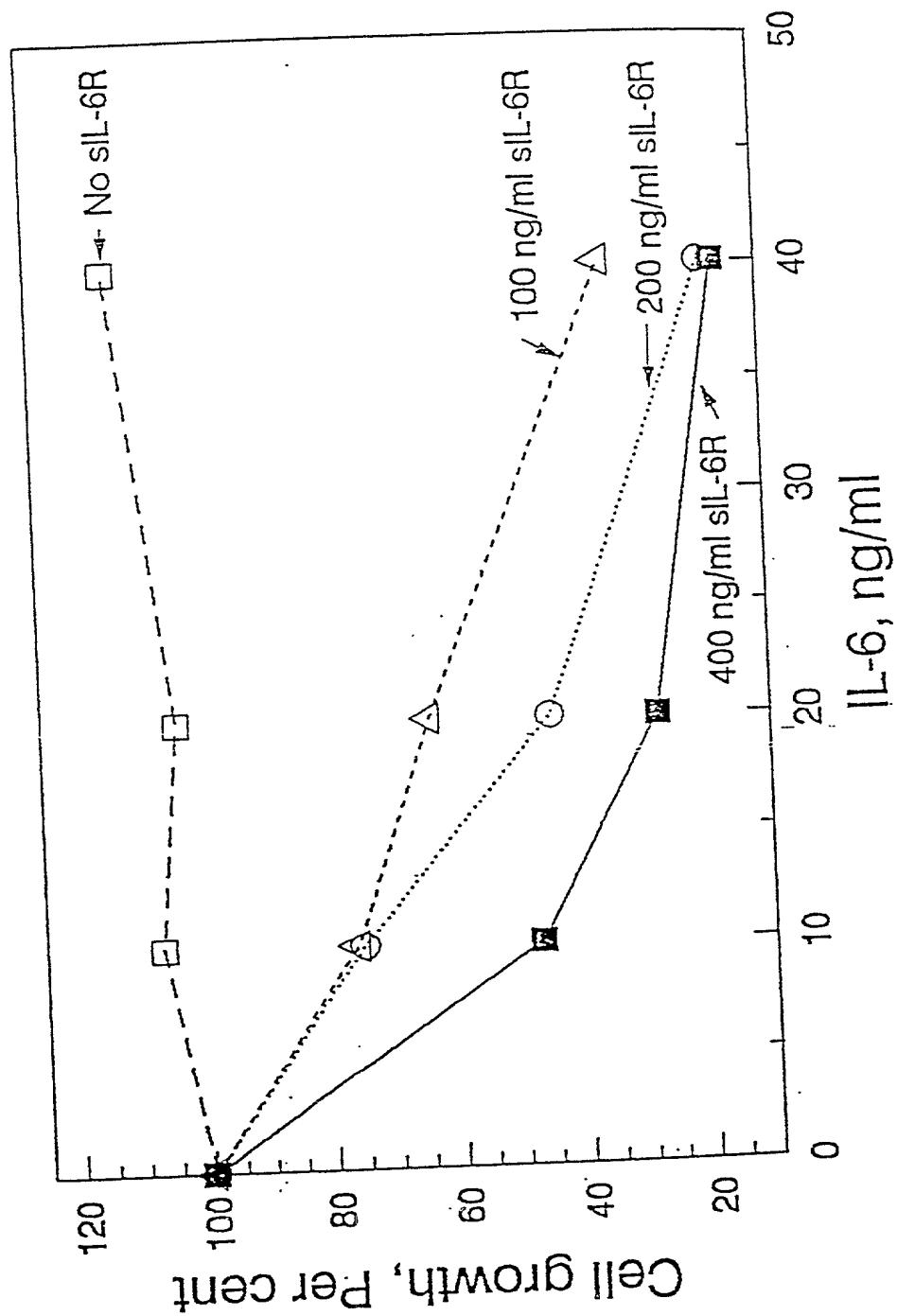


Fig. 6

09/462410

10/15

SCF, FLT-3	+	+	+	+	+
sIL6R/IL6 Chimera				+	+
IL-6, sIL-6R		+	+		

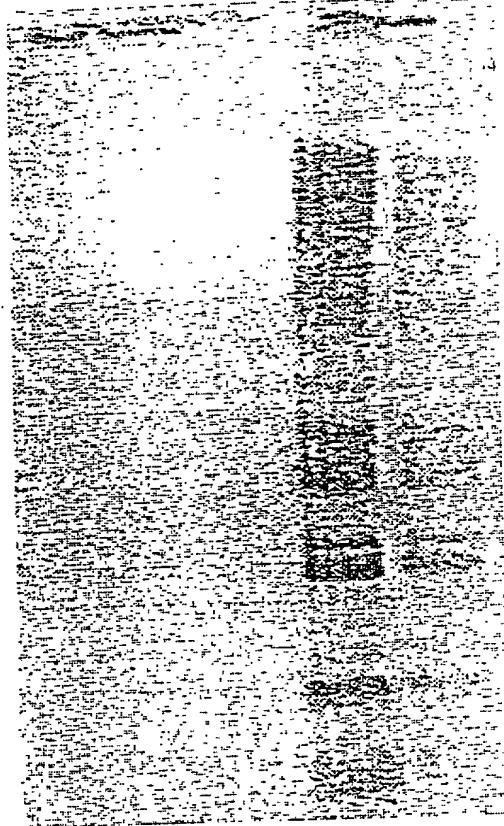


Fig. 7

09/462410

11/15

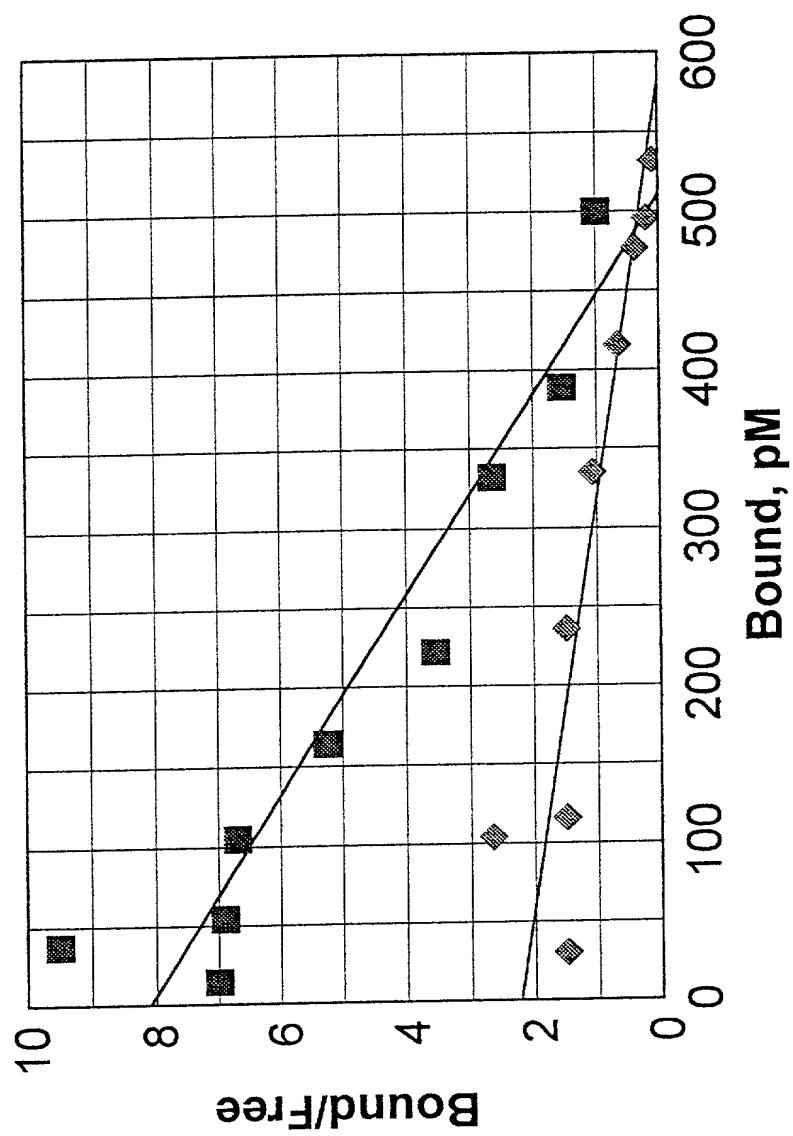


Fig. 8

09/462416

12/15

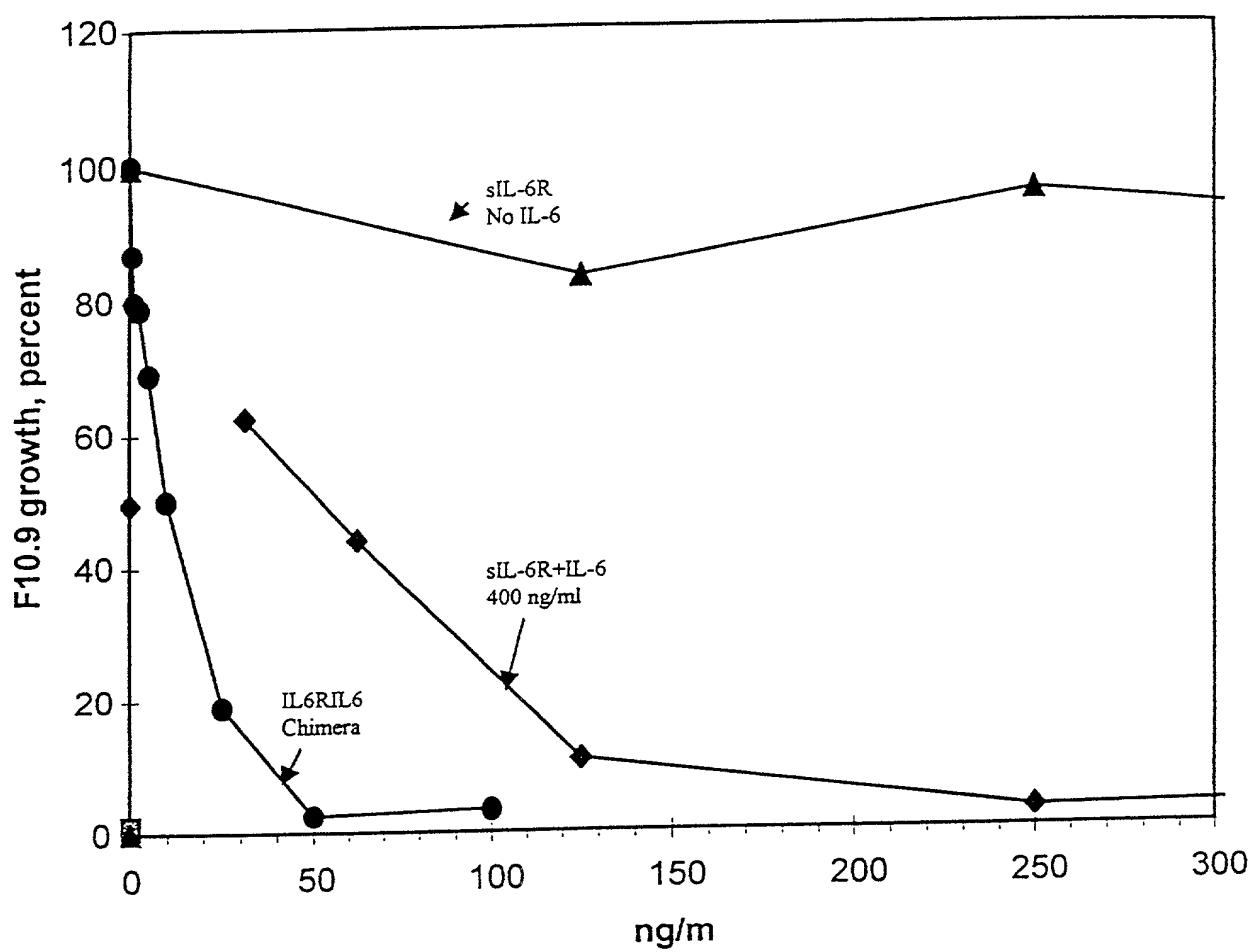


Fig. 9

09/462416

13/15

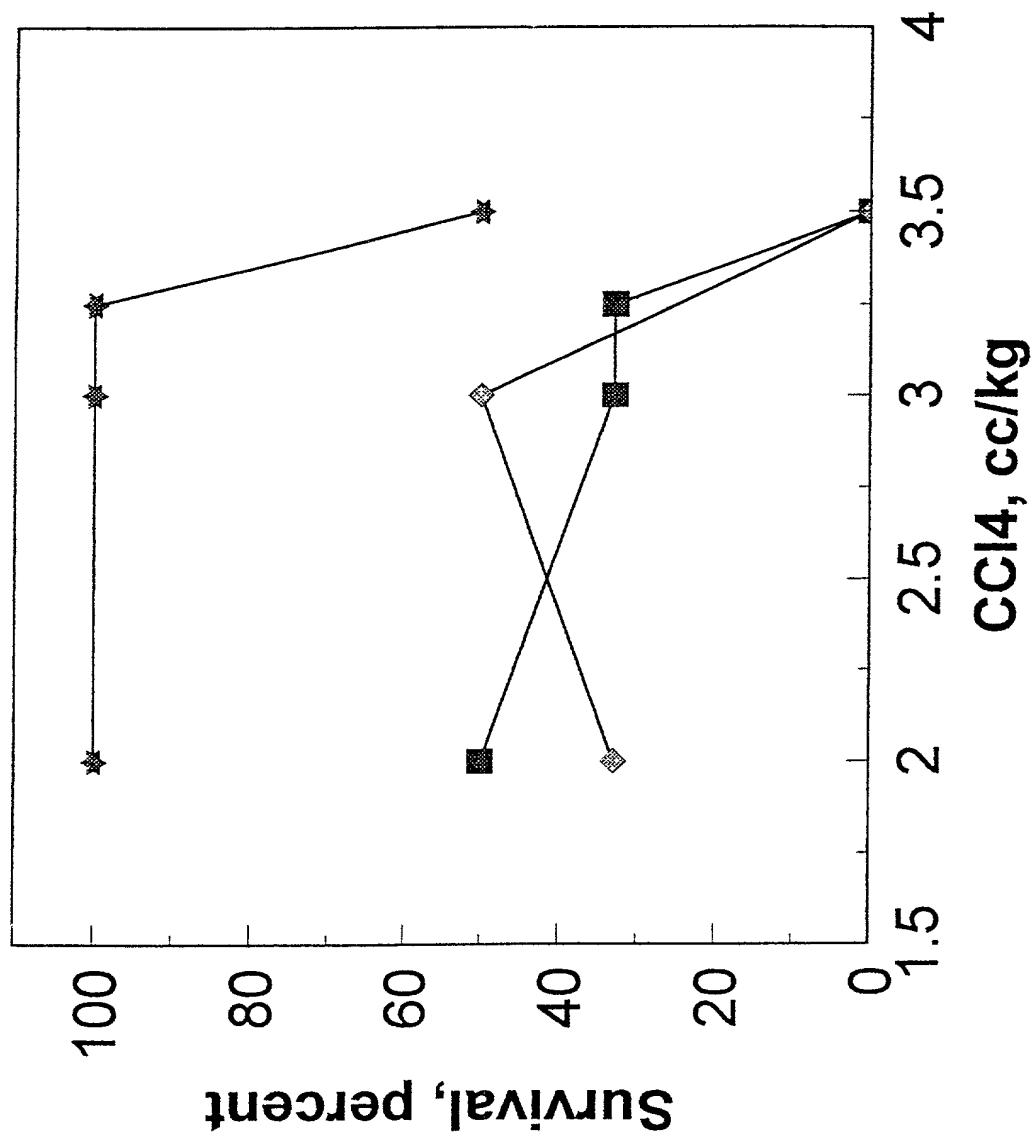


Fig. 10

09/462416

14/15

\* \* \* \* \*

1 MNSFSTSAGPVAFLGLLLVLPAAFPAPVPPGEDSKDVAAPHRQPLTSSERIDKQIRYI 60  
61 LDGISALRKETCNKSNCESSESKAELAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLL 120  
121 EFEVYLEYLQNRFESSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQ 180  
181 AQNQWLQDMTTHLILRSFKEFLQSSLRALRQMGGGGDPGGGGGPVPPEEPQLSCFRKS 240  
241 PLSNVVCEWGPRTSPSLTTKAVLLVRKFQNSPAEDFQEPCQYSQESQKFSCQLAVPEGDS 300  
301 SFYIVSMCVASSVGSKFSKTQTFQGCGILQPDPPANITVTAVARNPRWLSVTWQDPHSWN 360  
361 SSFYRLRFELRYRAERSKTFTTWMVKDLQHHCVIHDAWSGLRHVVQLRAQEEFGQGEWSE 420  
421 WSPEAMGTPWTESRSPPAENEVSTPMQALTTNKDDDNILFRDSANATSLPV\* 471

Fig. 11

09/462416

15/15

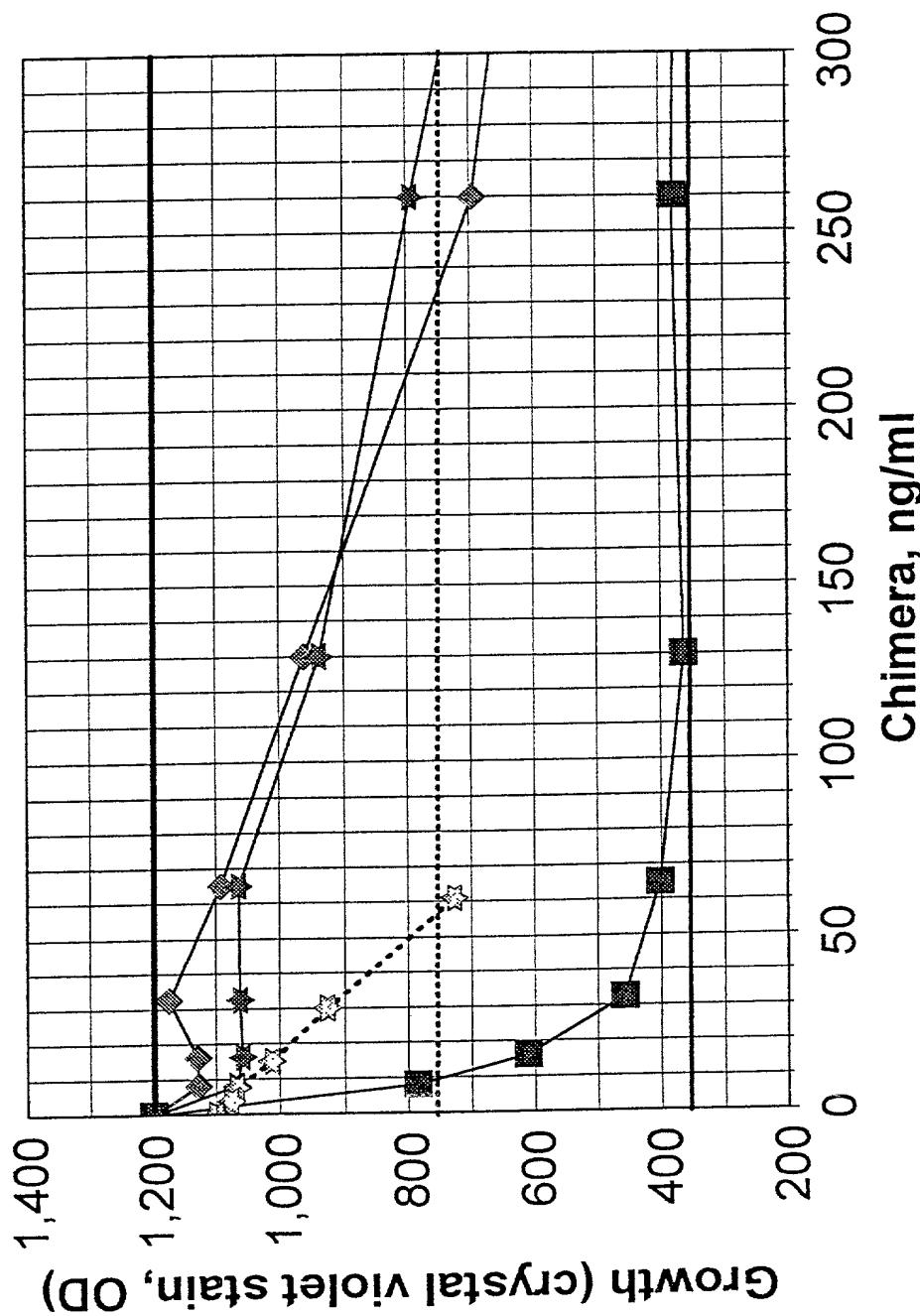


Fig. 12

### Combined Declaration for Patent Application and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Chimeric Interleukin-6 Soluble Receptor/Ligand Protein, Analogs Thereof and Uses Thereof

the specification of which (check one)

- [ ] is attached hereto;  
 [ ] was filed in the United States under 35 U.S.C. §111 on \_\_\_\_\_, as  
 U.S. Appln. No. \_\_\_\_\_ \*; or  
 [x] was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/IL98/00321; filed 9 July 1998, entry requested on 10 January 2000\*; national stage application received U.S. Appln. No. \_\_\_\_\_ \*; §371/§102(e) date \_\_\_\_\_ \* (\* if known)

and was amended on \_\_\_\_\_ (if applicable).

(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 and 365 of any prior foreign application(s) for patent or inventor's certificate, or prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked and have also identified below any such application having a filing date before that of the application on which priority is claimed:

121284 (Number)	Israel (Country)	10 July 1997 (Day Month Year Filed)	[x] YES	[] NO
122818 (Number)	Israel (Country)	30 December 1997 (Day Month Year Filed)	[x] YES	[] NO

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or prior PCT application(s) designating the U.S. listed below, or under §119(e) of any prior U.S. provisional applications listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information as defined in 37 C.F.R. §1.56(a) which occurred between the filing date of the prior application and the national filing date of this application:

(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

All of the practitioners associated with Customer Number 001444

Direct all correspondence to the address associated with Customer Number 001444, i.e.,

BROWDY AND NEIMARK, P.L.L.C.  
624 Ninth Street, N.W.  
Washington, D.C. 20001-5303  
(202) 628-5197

The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from Interlab Ltd. as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

Title: CHIMERIC INTERLEUKIN-6 SOLUBLE RECEPTOR/LIGAND PROTEIN...

U.S. Application filed \_\_\_\_\_, Serial No. \_\_\_\_\_

PCT Application filed 10 July 1998, Serial No. PCT/IL98/00321

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST INVENTOR <u>Michel REVEL</u>	INVENTOR'S SIGNATURE <i>Michel Revel</i>	DATE <i>Feb. 8, 2000</i>
RESIDENT Rehovot, Israel	<b>ILX</b>	CITIZENSHIP Israeli
POST OFFICE ADDRESS Weizmann Institute of Science, Beit Brazil 5, 76100 Rehovot, Israel		
FULL NAME OF SECOND JOINT INVENTOR <u>Judith CHEBATH</u>	INVENTOR'S SIGNATURE <i>Judith Chebath</i>	DATE <i>Feb. 14, 2000</i>
RESIDENT Rehovot, Israel	<b>ILX</b>	CITIZENSHIP Israeli
POST OFFICE ADDRESS Rehov Miller 13, 76284 Rehovot, Israel		
FULL NAME OF THIRD JOINT INVENTOR <u>Tsvee LAPIDOT</u>	INVENTOR'S SIGNATURE <i>Tsvee Lapidot</i>	DATE <i>Feb 14, 2000</i>
RESIDENT Ness-Ziona, Israel	<b>ILX</b>	CITIZENSHIP Israeli
POST OFFICE ADDRESS Rehov Boxer 6, 74046 Ness-Ziona, Israel		
FULL NAME OF FOURTH JOINT INVENTOR <u>Orit KOLLET</u>	INVENTOR'S SIGNATURE <i>Orit Kollet</i>	DATE <i>Feb. 14, 2000</i>
RESIDENT Ramat Gan, Israel	<b>ILX</b>	CITIZENSHIP Israeli
POST OFFICE ADDRESS Rehov Ramat Chen 14, 52232 Ramat Gan, Israel		
FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENT		
CITIZENSHIP		
POST OFFICE ADDRESS		
FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENT		
CITIZENSHIP		
POST OFFICE ADDRESS		
FULL NAME OF SEVENTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENT		
CITIZENSHIP		
POST OFFICE ADDRESS		